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"Nitric Oxide Releasing Multitarget Drugs"

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CHAPTER 1

*NITRIC OXIDE RELEASING HYBRIDS OF CARDIOVASCULAR DRUGS**

*The contents of chapter 1 have been already published:

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Introduction

The pharmacological treatment of many pathological states often requires the cooperation of different and complementary pharmacodynamic mechanisms. In the clinical practice, this is frequently achieved through the administration of "cocktails", composed by more drugs possessing different mechanisms of action.

In the last years, the "one-drug-one-target" paradigm has been shelved and numerous multi-target drugs have been projected and synthesised (Fig. 1). Such compounds share two (or more) desired pharmacodynamic properties, ensured by the presence of overlapping or conjugated pharmacophores.

Compared with a pharmacological "cocktail", the use of only one multi-target drug presents some advantages, such as an easier prediction of pharmacokinetic/pharmacodynamic relationships and an improved compliance by the patient, due to the administration of a single medicine (Morphy et al., 2004).

The release of nitric oxide (NO) is the mechanism of action accounting for the pharmacological features and clinical applications of drugs, such as the "old" vasodilators nitrites and nitrates, because of the fundamental roles played by this small molecule in the cardiovascular system. More recently, the NO-releasing ability has been individuated as an interesting property to be added to molecules already possessing another pharmacodynamic pattern.

This strategy led to the development of important multi-target drugs, interesting pharmacodynamic hybrids joining to a "native" mechanism of action a NO-donor property, addressed to reduce possible side-effects (for example, the gastrotoxicity of aspirin) or improve the therapeutic impact (e.g., the increase of antiplatelet activity of aspirin, again).

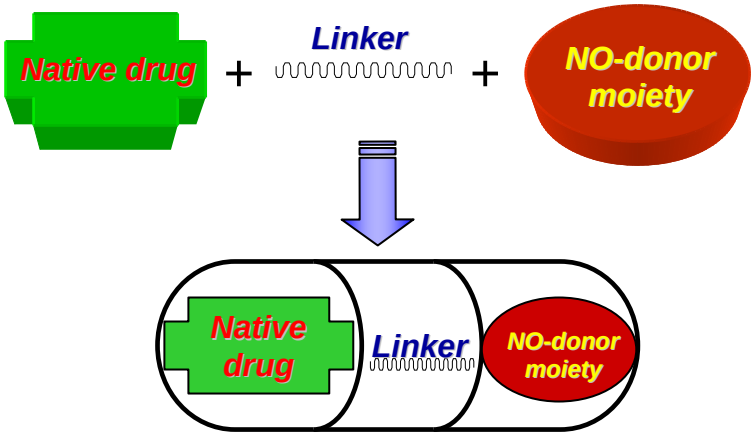


Figure 1. Theoretical template for the synthesis of a pharmacodynamic hybrid. A NO-releasing function is generally bound to a "native" drug, directly or often through a linker moiety.

This PhD work focuses on the fundamental biological properties of NO at the cardiovascular level, on the chemical features of the most commonly used NO-donor moieties involved in the mechanisms ensuring the release of NO, and on the main classes of multi-target drugs, showing the conjugation of a NO-donor group to a "native" molecule acting at the cardiovascular level.

Endothelial Nitric Oxide

The vascular endothelium plays a fundamental role in the regulation of several aspects of blood circulation. Following both mechanical and chemical stimuli, it biosynthesises and releases many endogenous compounds, which control and modulate the tone of vascular smooth muscle cells, their proliferation and several steps involved in the process of emostasis. In many pathological states, endothelial disfunction is known to account for several aspects involved in the pathogenesis of cardiovascular disorders (Noll and Lüscher, 1998). Among the heterogeneous compounds produced by endothelial cells, the most relevant one is

probably a small molecule, originally described as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) identified as nitric oxide (Palmer et al., 1987; Ignarro et al., 1987; Myers et al., 1990). In the endothelial cell, NO is biosynthesised by the endothelial Ca^{2+}-dependent constitutive enzyme NO-synthase (e-NOS) from L-arginine (Fig. 2) (Palmer et al., 1988).

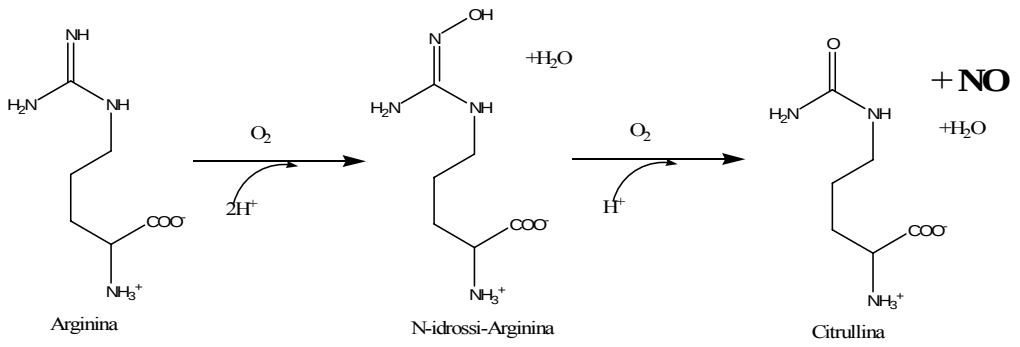


Figure 2. Biosynthesis of nitric oxide by NOS from L-arginine.

Besides the well-known endogenous and exogenous compounds (such as acetylcholine, bradykinin, calcium ionophore A23187, etc.) accounting for a chemical stimulation of the release of endothelial NO, blood flow and shear stress are the mechanical physiological triggers for the production and release of endothelial NO (Pohl et al., 1986; Rubanyi et al., 1986) and an endothelium-mediated vasorelaxing effect induced by blood flow has been demonstrated in animal and human vessels (Hintze and Vatner, 1984; Hull et al., 1986; Pohl et al., 1986; Miller and Vanhoutte, 1988; Drexler et al., 1989).

Conversely, the inhibition of eNOS causes vasocontractile effects (Joannides et al., 1995; Reesrees et al., 1990; Yang et al., 1991; Tschudi et al., 1991) and determines a significant reduction of perfusion flow in isolated perfused tissues (Meyer et al., 1993). This endothelium-mediated vasorelaxing effect is principally due to the NO-induced activation of cytosolic guanylate cyclase in the vascular

smooth muscle cell, with a consequent raise of intracellular concentration of cGMP (Rapoport et al., 1983; Alonso and Radomski, 2003) (Fig. 3). Besides, other vasorelaxing mechanisms, such as a direct activation of muscular potassium channels by NO (Bolotina et al., 1994), have been described.

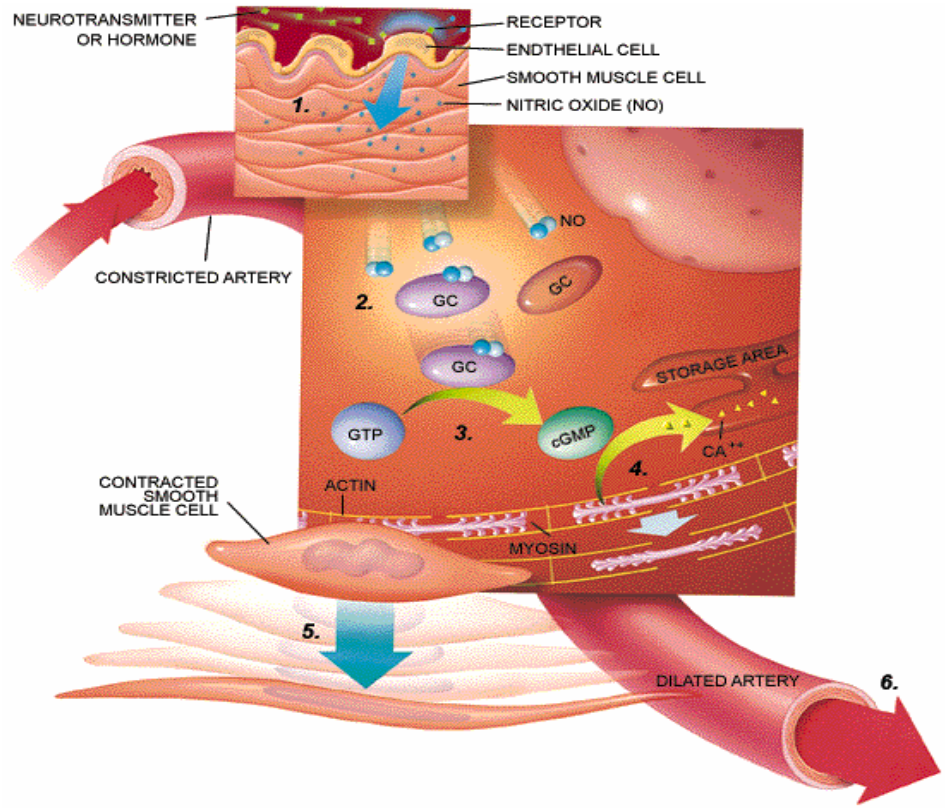


Figure 3. Nitric oxide pathway in vascular and endothelial district.

To remark the fundamental role played by the vasorelaxing effect of endothelial NO in the regulation of blood pressure within the physiological range, it is noteworthy that often most cardiovascular diseases are associated with an impairment of the vasodilator function of endothelium (Vanhoutte, 1989; Mombouli and Vanhoutte, 1999; Vanhoutte and Boulanger, 1995). Indeed, the NO-dependent vasorelaxing effects of acetylcholine are decreased in patient affected by essential hypertension, as well as in aged humans (Panza et al., 1990; Cardillo and Panza, 1998; Taddei et al., 1995; Taddei et al., 1997). Furthermore,

specific physiological functions of particular vascular districts, such as the *corpus cavernosum,* are ensured by NO, whose release induced by NANC (non-adrenergic non-cholinergic) neural influence, with the consequent vasorelaxing effects, is a key-step involved in penis erection (Rajfer et al., 1992). Besides its fundamental vasorelaxing effect, NO controls an another important aspect of cardiovascular system: the platelet function. In particular, NO, produced by the endothelial cells but also by the platelet themselves, reduces the platelet adhesion and aggregation (Radomski et al., 1990; Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987).

NO is also involved in the regulation of the modelling of the vascular structure, through both direct and indirect mechanisms. Indeed, the platelet adhesion on the site of an endothelial lesion determines the proliferation of vascular smooth muscle cells, due to the release of platelet-derived factors (Ross, 1993). Therefore, the anti-platelet function of NO represents *per se* an indirect anti-proliferative function. Furthermore, NO inhibits the biosynthesis of MCP-1 (monocytes chemoattractive protein) and, consequently the adhesion of monocytes to the vascular wall, where they could release proliferative factors and cytokines (Hannan et al., 1988). More recently, a possible role of NO in the process of "ischemic preconditioning" has been extensively debated. Although, to date, the different experimental approaches on different animal species do not allow a consistent unitary theory on the exact role played by endogenous NO and on the specific contributes of the different isoforms of NO synthase (Schulz et al., 2004; Lochner et al., 2000), there are clear and unequivocal evidences that the administration of exogenous NO-donors determines a significant reduction of the

myocardial damage in ischemia-injured hearts from different animal species (Qin et al., 2004; Masini et al., 1999; Nakano et al., 2000; Schlack et al., 1995; Pabla et al., 1995). This experimental evidence lets us foresee a further intriguing positive aspect for a rationale use of NO-releasing molecules as potential anti-ischemic drugs.

NO-donor drugs

The prototypical class of NO-releasing derivatives is represented by organic nitrates and nitrites, such as glyceryl trinitrate, isosorbide dinitrate or 5-mononitrate and amyl nitrite, able to release NO after a metabolic bio-transformation, or other molecules able to release spontaneously NO with a temperature dependent mechanism, such as sodium nitroprusside (Fig. 4).

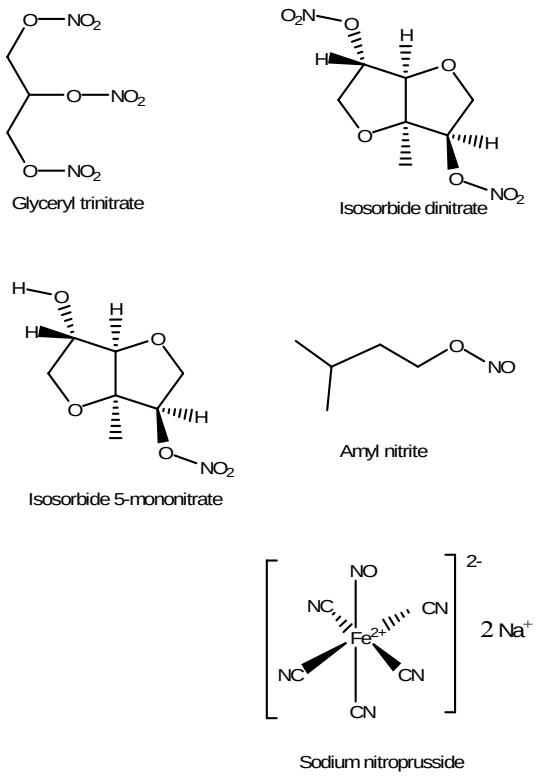


Figure 4. Chemical structures of the "classical" NO-donors glyceryl trinitrate, isosorbide dinitrate, isosorbide 5-mononitrate, amyl nitrite and sodium nitroprusside.

All these compounds can be viewed as pro-drugs, which, through the release of exogenous NO, activate the same metabolic pathway of endogenous NO (Torfgard and Ahlner, 1994) and thus can exhibit all its biological properties. Nevertheless, their use is substantially confined into the only sphere of pathological situations requiring a rapid significant vasorelaxing effect, because of their short half-life (i.e. a rapid and massive release of NO).

In the last two decades, two different biochemical activations for organic nitrates were hypothesized and then described: the enzymatic and the non-enzymatic ones. The enzymatic pathway, has its primary location on plasma membrane of muscular or endothelial cells (Fung et al., 1992).

Although the enzymes responsible for the *in vivo* denitration and reduction of organic nitrates have not still identified, different potential enzymes have been purposed for this role, such as glutathione-*S*-transferase (Kurz et al., 1993), cytochrome P-450 like enzymes (McGuire et al., 1998), and according to recent studies, also mitochondrial aldehyde dehydrogenase (ALDH2) seems able to mediate denitration of classical nitrates (Chen et al., 2002). Glutathione-*S*-transferase is responsible for the conversion of glyceryl trinitrate (GTN) in its metabolites 1,2-GDN and 1,3-GDN (Lau et al., 1990). Such enzyme probably uses the reduced thiolic group of glutathione, which is bound to the catalityc active site, to release nitroso acid and the glutathione oxidated form. The nitroso acid then is reduced to NO, by protonation, or by the formation of of S-nitrosothiol (Ignarro, 1989). As concerns the non-enzymatic pathway, in the late ’60, Needleman and coworkers (Needleman and Krantz, 1964; Needleman et al., 1969) introduced the concept of organic nitrates as “prodrugs” which need

intermediates to act their mechanism of action. According to this hypothesis, molecules containing SH groups, such as cysteine or glutathione, appeared indispensable for the conversion of organic nitrates into NO or S-nitrosothiols (Ignarro et al., 1981). Today it is suggested that organic nitrates, interacting with sulphydryl groups, produce NO or *S*-nitrosothiols; these intermediates, activating guanylate cyclase, produce cGMP which determines vasodilatation.

A recent study focuses the attention on the mechanism of cytochrome P450 reductase (CYP450R)-mediated nitric oxide and nitrosothiol generation from organic nitrates. According to this study the cytochrome P450 reductase catalyzes the bioactivation of organic nitrate through reduction to form the intermediate organic nitrite, which is converted to NO and nitrosothiols in a thiol-dependent reaction. This series of experiments was performed both on rat liver microsomes (containing the CYP450R-CYP450 complex) and on purified recombinant CYP450R. The presence of NADPH, compared with NADH, results in a much more efficient reducing substrate, as electron donor, to support the CYP450R-mediated GTN/isosorbide dinitrate (ISDN) reduction to produce nitrite, thanks to its better substrate affinity for CYP450R. The CYP450R flavin site inhibitor, diphenyleneiodonium, inhibits the NO_2^- generation, whereas the CYP450 inhibitor clotrimazole doesn't inhibit this first step but gretly inhibits NO_2^--dependent NO generation. Therefore, CYP450R catalyzes organic nitrate reduction, producing nitrite, whereas CYP450 seems to mediate further nitrite reduction to NO. However nitrite-dependent NO generation contributed <10% of the CYP450R-CYP450 mediated NO generation from organic nitrates suggesting that nitrite is not the primary precursor of NO in the process of microsomal

CYP450R-CYP450-mediated organic nitrate biotransformation but is the precursor of both NO and nitrosothiols. In fact it is well known that sulphydryl compounds are needed in GTN activation and that the repeated administration of GTN causes sulphydryl depletion and consequent tolerance to further vasodilatation. This study shows that the presence of L-cysteine triggered significant NO generation from recombinant CYP450R-mediated reduction of GTN/ISDN, whereas no detectable NO was generated without the addition of thiols. While, with microsomal CYP450R, external thiols were not required for NO generation, as the sulphydryl groups in the microsomal proteins may serve to reduce organic nitrite to NO. So the proposed mechanism of CYP450R-CYP450-mediated biotransformationof organic nitrate is the following:

1° Step

$$R\text{-}O\text{-}NO_2 + NADPH + H^+ \xrightarrow{\textbf{CYP450R}} R\text{-}O\text{-}NO + NADP^+ + H_2O$$

2° Step

$$R\text{-}O\text{-}NO + H_2O \longrightarrow R\text{-}O\text{-}H + HNO_2$$

3° Step

$$HNO_2 + H^+ \xrightarrow{\textbf{CYP450}} NO + H_2O$$

4° Step

$$2R\text{-}O\text{-}NO + 2R'\text{-}SH \longrightarrow 2NO + 2ROH + R'\text{-}S\text{-}S\text{-}R'$$

5° Step

$$R\text{-}O\text{-}NO + R'\text{-}SH \longrightarrow R\text{-}OH + R'\text{-}S\text{-}NO$$

According to this mechanism organic nitrite (R-O-NO) is the initial product in the process of CYP450R-mediated organic nitrate biotransformation and is the precursor of NO and nitrosothiol. In the absence of thiols, organic nitrite

undergoes hydrolysis to form nitrite. However in the presence of thiols, either NO or nitrosothiols can be formed (Li et al., 2006).

Besides the organic nitrates and nitrites, which require a metabolic biotransformation for activity (Parrat, 1979), there are direct NO-donors able to release NO spontaneously from a nitroso or nitrosyl moiety.

Although metabolic processes, due to NADPH-generating systems located in the cell membrane, seem to be involved in an increased release of NO from sodium nitroprusside (SNP), these "facilitating" processes are not required as a necessary condition for the NO-release (Kowaluk et al., 1992). Therefore SNP is generally classified as an agent belonging to the class of spontaneous NO-donors (Ignarro et al., 1999; Ignarro et al., 2002a; Thatcher et al., 2004). In its molecule (Fig. 4), a square bipyramidal structure, a nitrosyl group is linked to a ferrous ion connected also with five cyanide anions (Ignarro et al. 1999). NO is released from this complex at physiological pH. However, the clinical employment of this drug, as a vasodilator, is limited by the toxicity due to the formation of thiocyanate and the need of parenteral administration (Ignarro et al., 2002). Moreover, SNP exhibits the induction of an enhanced hydrogen peroxide-mediated cytotoxicity, probably due to the release of cyanide and intracellular residual iron complexes (Wink et al., 1996).

Diazeniumdiolates, also called NONOates (Fig. 3), possess the ability to release directly NO as a radical. Their efficacy was demonstrated in reversing cerebral vasospasm, reducing pulmonary vascular pressure and their coupling with metallic stents is studied in order to reduce the risk of restenosis following angioplasty surgical interventions (Keefer, 2003).

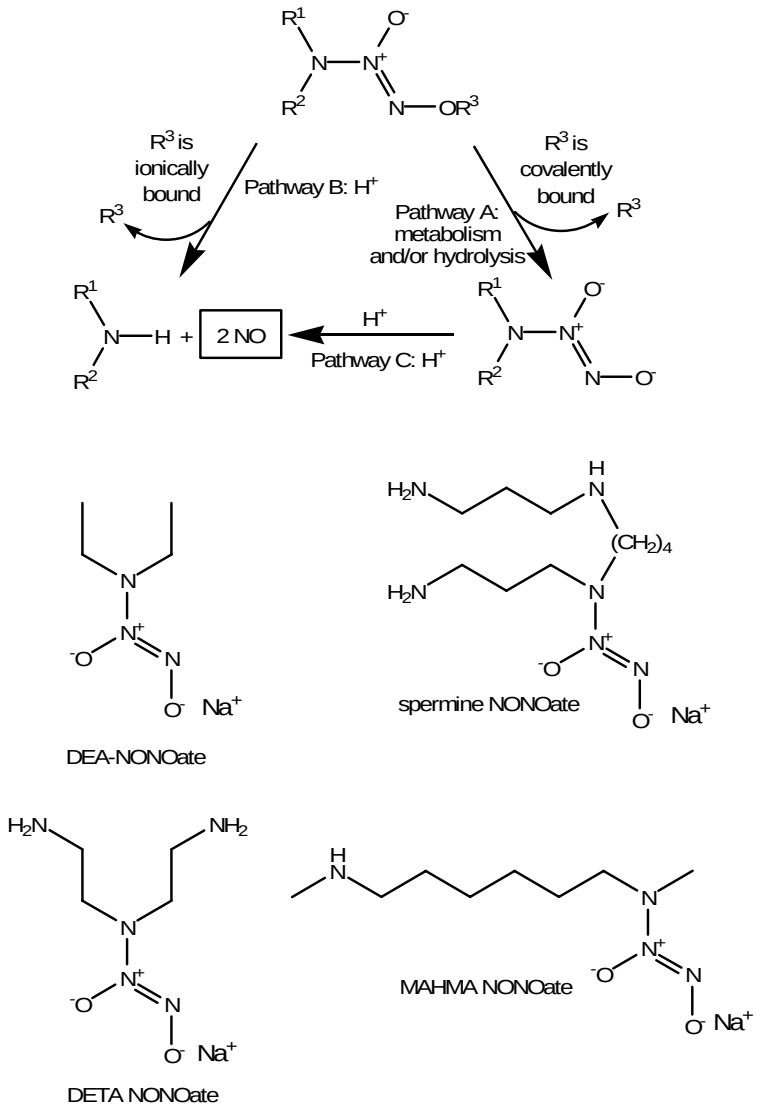
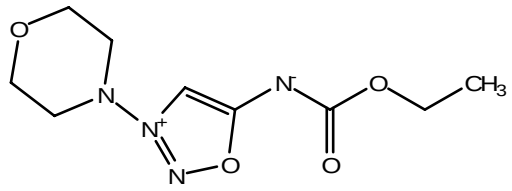


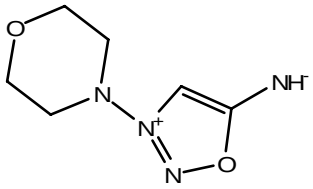
Figure 5. The two mechanisms accounting for the release of NO from a generic diazeniumdiolate (NONOate): if the O-R^3 bond is ionic, a H^+-catalysed reaction leads to the release of NO (pathway A); if the O-R^3 bond is covalent, a first biotransformation leads to an intermediate derivative (pathway B), which in turn is converted into NO by a H^+-catalysed reaction (pathway C). The chemical structures of some NONOates, commonly used as NO-donors, are also shown.

Among the direct NO-donors there are two classes of heterocyclic NO-releasing drugs, furoxanes and sydnonimines (Fig. 6), which require a facilitator agent to release NO. These cofactors are represented by thiol-derivatives for furoxanes and by oxidants for sydnonimines. The most known sydnonimine, the 3-

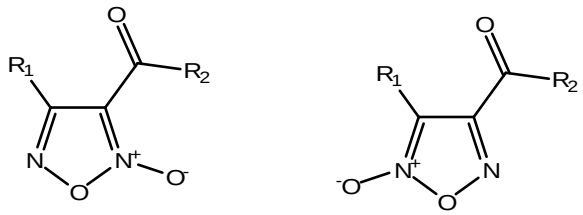
morpholinosydnonimine (SIN-1), deriving from molsidomine, represents the combination of morpholine and a sydnonimine; it has been hypothesised that this zwitterionic compound, at physiological pH, decomposes into $NO^{\cdot}$ and superoxide anion (Feelisch et al., 1998). Nevertheless, other theories suggest that the composition of SIN-1 could lead to nitrogen oxide products, other than NO; these products (such as peroxynitrite) could be responsible for toxicity (Wink et al., 1996).



Molsidomine



3-Morpholino sydnonimine (SIN-1)



Furoxan derivatives

Figure 6. NO-releasing heterocyclic moieties: molsidomine, SIN-1 and generic structures of furoxanes.

As concerns furoxanes (1,2,5-oxadiazole 2-oxide derivatives), they are often employed as NO-donor moieties linked to "native" drugs in order to add cGMP-

mediated vasodilating and antiplatelet properties (Ghigo et al., 1992; Feelisch et al., 1992).

Finally, the S-nitrosothiols must be mentioned, a class of NO-donating compounds which represent a source of circulating endogenous NO releasing spontaneously NO and NO^+ (nitrosonium) (Napoli and Ignarro, 2003).

S-nitrosothiols may also be able to improve transmembrane transfer of NO, realised by the cell-surface protein disulfide isomerase (PDI) from a S-nitrosothiol source (Zai et al., 1999). Moreover, S-nitrosothiols seem to be involved as intermediates, in the organic nitrate mechanism of action. In fact, organic nitrates first interact with sulphydryl groups to produce NO (or S-nitrosothiols) which in turn activate guanylate cyclase to produce cGMP (which causes vasodilation). This biochemical cascade, albeit not indispensable for the mechanism of nitrates, seems to be chemically very favourable and rapid (Fung et al., 1992). Besides, members of this class, as S-nitroso-N-acetylpenicillamine (SNAP), show some advantages with respect to organic nitrates or SNP, in fact they exhibit a lower capacity to induce oxidative stress or tolerance in vascular cells (Jaworski et al., 2001).

Indeed, the induction of tolerance seems to be one of the most relevant problems, related with a long-term administration of many NO-releasing drugs. Although the comprehension of the exact mechanisms accounting for the development of such a tolerance is still controversial, it is accepted that this represents a multifactorial phenomenon involving several (and not completely clarified) processes. Besides an early hypo-responsiveness (known as pseudotolerance), mainly due to counter-regulatory mechanisms, such as an increased production of

endogenous vasoactive compounds, a long-term administration of organic nitrates, and in particular glyceryl trinitrate, has been related with many intrinsic alterations, leading to a more significant loss of responsiveness (tolerance) (Fung, 1987; Fung and Bauer, 1994; Munzel et al., 1996; Parker et al., 1991; Abdollah et al., 1987; Parker and Parker, 1998; Gori and Parker, 2002a; Gori and Parker, 2002b; Fung, 2004; Munzel et al., 2005).

More in general, the tolerance to a NO-releasing agent is a complex phenomenon, probably linked to the involvement of several aspects, depending both on the chemical nature of the drug and on the triggering of "proximal" and "distal" biological mechanisms. The "proximal" events are those related to the alteration of one or more steps possibly involved in the bio-transformation of the drug, leading to the release of NO, but also those related to the metabolic fate of the released NO. Instead, the "distal" mechanisms are those more closely related to the possible reduction of the NO-induced rise of cGMP.

As concerns the "proximal" ones, the induction of tolerance by organic nitrates has been studied, taking glyceryl trinitrate as the most commonly used NO-donor reference compound. A significant role of a decreased activity of enzymes, such as aldehyde deydrogenase 2 (ALDH2) and cytochrome P-450 (both probably involved in the bio-activation of glyceryl trinitrate), has been suggested as a cause of tolerance. Indeed, ALDH activity was reduced in *in vitro* models of vascular tolerance and ALDH-inhibiting drugs could produce a reduced response to glyceryl trinitrate (Chen et al., 2002). However, other *in vivo* studies led to controversial results (Di Fabio et al., 2003), indicating that a decreased enzymatic activity of ALDH2 can play a role in the development of tolerance, but this role is

not dominant. Instead, an important role played by a decreased activity of cytochrome P-450 has been proposed, because the pharmacological induction of this enzymatic system could reverse the development of tolerance caused by glyceryl trinitrate (Minamiyama et al., 2001), while a vitamin E-deficiency, related to a decrease of cytochrome P-450 expression (but also to an increased oxidative stress) caused an acceleration in the development of tolerance to glyceryl trinitrate (Minamiyama et al., 2006). Nitrate tolerance seems to derive, at least in part, also from an intracellular sulphydryl depletion; this event reduces the metabolic conversion of organic nitrates to NO and thereby vasodilation (Fung et al., 1992). But some experimental evidences show that non-sulphydryl compounds, like enalapril (Katz et al., 1991) and hydralazine (Bauer and Fung, 1991) can reverse or prevent the occurrence of *in vivo* nitroglycerine-induced tolerance, indicating that the sulphydryl depletion-theory itself is not sufficient to explain the mechanisms of nitrate tolerance. Of course, the development of tolerance mechanisms, related to a reduced bio-activation of organic nitrates, can satisfactorily explain the cross-tolerance between two drugs which share a common metabolic activation pathway. On the contrary, this can not account for the cross-tolerance observed also between an organic nitrate and a spontaneous NO-donor, (or a drug able to release NO through different activation pathway). For example, a reduced responsiveness to SIN-1 has been observed in vascular preparations obtained from rabbits in which tolerance was induced by glyceryl trinitrate (Daiber et al., 2005). Moreover, in the “tolerant” vessels, the authors found high levels of superoxide levels and increased amounts of nitrotyrosine, a stable metabolite of peroxynitrite. This experimental evidence led to hypothesise a

significant role of an accelerated inactivation of NO, as a possible cause of such a cross-tolerance (Daiber et al., 2005). Indeed, increased levels of superoxide and of peroxynitrite (readly produced by the reaction of surperoxide with NO) have been observed in rat vessels made tolerant by glyceryl trinitrate. Consistently, tolerance was reduced by pre-treatment with several antioxidant, such as ascorbic acid and uric acid (Abou-Mohamed et al., 2004). Finally, also the "distal" mechanisms, such as a decreased enzymatic activity of guanylate cyclase (Axelsson and Ahlner, 1987) and/or an increased hydrolytic degradation of cGMP (Kim et al., 2001), can lead to a hypo-responsiveness to NO-donors. Of course, such mechanisms can perfectly participate to the multifactorial phenomenon of tolerance to organic nitrate and can also explain the tolerance observed for spontaneous NO-releasing agents. Indeed, even if the tolerance induced by non-nitrate NO-donors is generally considered to be of lower magnitude with respect to that induced by organic nitrates, relatively high concentrations (0.1-10 μM) of SNP determined a significant desensitisation of soluble guanilate cyclase and the development of cross-tolerance with authentic NO (Sorajja et al., 2005). However, the development of this desensitisation was not observed after treatment with lower concentrations (1 nM) of SNP (Sorajja et al., 2005). It is interesting to note that some particular effects of NO-donor drugs, such as the anti-platelet one, seems to be resistant to the development of tolerance (Holmes et al., 2005).

The problem of tolerance for NO-donors, whose whole comprehension needs further experimental data, has been authoritatively reviewed by some excellent papers (Thatcher et al., 2004; Papapetropoulos et al., 1998).

NO-donor hybrids

In the last years, the knowledge of the biochemical and pharmacological properties of nitric oxide led to the effort in design of new hybrid molecules, which couple a "native" well-known drug with a NO-donor moiety (Fig. 5). This innovative approach furnished a variegated sample of new chemical entities which conserve the therapeutic efficacy of the "parent" drug, enriched through the nitric oxide activity, moreover this "synergism" often resulted in a reverse of their side effects.

Among the NO-donor hybrids an increasing interest was aroused by the NO-releasing cardiovascular drugs, such as aspirin, statins, α and β-blockers, Ca^{2+}-antagonists, K_{ATP}-openers, ACE-inhibitors and sartans; the goal of these hybrid molecules is the improvement of their cardiovascular actions and, sometimes, even the elision of their adverse effects thanks to NO properties.

NO-aspirins

One of the most characterised classes of NO-donors hybrids is represented by NO-NSAIDs (NO-donating non steroidal anti-inflammatory drugs) which, synthesised by esterifiation of the parent drug (a NSAIDs drug) with a NO-donor chain, maintain the original anti-inflammatory properties and show, through the nitric oxide action, a marked reduction of gastrolesivity. In fact, NO determines a protective effect on gastric mucosa attributable to several pharmacodynamic mechanism such as the assurance of mucosal blood flow, the stimulation of the mucus production, the inhibition of leukocytary adhesion and of enzymatic

caspase (Chiroli et al., 2003; Wallace et al., 1994; Wallace et al., 1995; Moncada et al., 1991).

As concerns the pharmacokinetic, the NO-NSAIDs may be assimilated to organic nitrates because they also require metabolic activation before releasing NO. Although the enzymes involved are not yet identified, there are a lot of evidences about a role of esterases in the cleavage of NO from the parent drug (Burgaud et al., 2002; Cirino et al., 1995; Keeble et al., 2001). Nevertheless, even if the involvement of esterases is compatible with the chemical structure of NO-NSAIDs (the NO-releasing moiety is linked to the "native" molecule by an ester bond) also cytochrome P450 seems to act in the NO-NSAIDs breakdown (Grosser and Schroder, 2000). Moreover, several different experiments on the rate of the enzymatic catabolism, both *in vitro* and *in vivo*, indicate that NO-NSAIDs have a NO-release slower than the "classical" NO-donors, such as SNP or SNAP (Santini et al., 1996). This gradual release may be the key to explain why, in experimental animals, NO-NSAIDs do not alter systemic arterial blood pressure even when administered intravenously, in large dose, whereas an equimolar dose of the "classical" NO-donors causes a deep hypotension (Burgaud et al., 2002).

A recent study of Knaus' group shows the development and the biological evaluation of a new serie of NO-NSAIDs. Hybrid NO-NSAIDs possessing a 1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolate or 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate moiety linked by a one-carbon methylene spacer to the carboxylic acid group of "native" aspirin (Fig. 6), ibuprofen and indomethacin were synthesized and characterised as NSAID and NO-donors. Although these new molecules did not show any inhibitory activity against COX_1 and COX_2, they

result in a decreasing of carrageenan-induced rat paw edema similar to that exhibited by the "native" drugs and in improving of classical NSAIDs ulcerogenity.

As regards NO-release an increased effect was observed in tests carried out in presence of guinea pig serum with respect to those in phosphate buffer solution.

All these considerations seem to suggest that such compounds are prodrugs which require a metabolic activation reaction (esterase-mediated ester cleavage) to be active (Velázquez et al., 2005).

The NO-releasing aspirin derivatives represent a new class of nitric esters in which a nitrate group is coupled to the carboxylic moiety of acetylsalicylic acid (ASA) through a variety of spacers (aliphatic, aromatic or heterocyclic ones) (Fig. 7) which result in conferring different pharmacokinetic or pharmacodynamic properties to the new hybrid (Chiroli et al., 2003). The nitroaspirin NCX 4016 [3-(nitrooxymethyl)-phenyl 2-(acetyloxy)-benzoate] is the lead compound of this new category of anti-inflammatory and antithrombotic drugs (Del Soldato et al., 1999) because this chimerical compound is able to maintain the anti-inflammatory action without the gastrointestinal toxicity and to show new cardiovascular properties due to the NO-donor moiety.

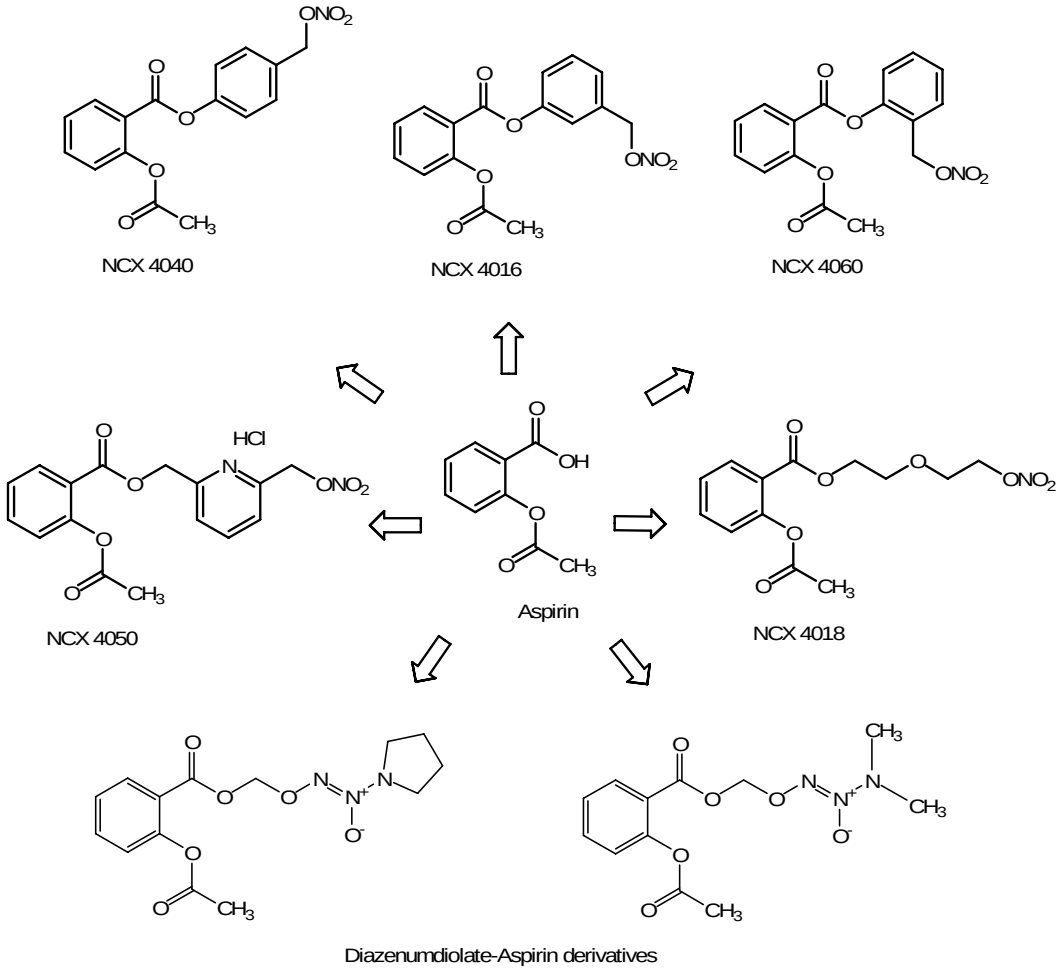


Figure 7. Structures of several NO-releasing hybrids, obtained from the "native" drug aspirin. The hydrolytic cleavage of the linkers (carrying the NO-donor moiety) leads to the release of the "native" drug, without any structural alteration.

Aspirin is the most known NSAID but also its use for the prevention and the treatment of such cardiovascular diseases, such as thrombosis, is well established (Harter et al., 1979; Hirsh, 1979). The anti-thrombotic effect of aspirin is due to the irreversible inhibition of COX-1 which determines a reduction in the production of the pro-aggregatory TxA_2 by platelets and of the anti-aggregatory PGI_2 by vascular endothelial cells, but while the endothelial cells are able to synthesise new COX-1 enzyme, platelets do not show this possibility, therefore the final balance is shifted towards the inhibition of platelet aggregation (Schror,

1997). However, aspirin blocks only one pathway of platelet activation thereby leaving the others unaffected and this may limit its effectiveness like antithrombotic agent (Gresele and Agnelli, 2002; Folts et al., 1999).

NO is well known as anti-aggregatory agent, in fact it suppresses platelet activation by activating guanylate cyclase (GC), thereby increasing the conversion of GTP to cGMP, enhancing calcium ATPase-dependent refilling of intracellular calcium stores and inhibiting the activation of phosphoinositide 3-kinase (PI3K). As a consequence, intracellular calcium (Ca^{2+}) flux is inhibited, leading to suppression of P-selectin expression and of the active conformation of glycoprotein IIb/IIIa (GPIIb/IIIa) required for binding fibrinogen (Loscalzo, 2001).

Hence, nitroaspirin could represent a good solution for ASA lacks, because its mechanism of action results from the sum of COX-mediated effects, such as inhibition of biosynthesis of inflammatory prostaglandins and of platelet aggregation, and NO-mediated effects (cGMP-dependent and independent) such as vasodilation, inhibition of platelet aggregation, leukocyte adherence to the vascular endothelium, inflammatory cytokine synthesis (through nitrosylation of caspase) and apoptosis (through nitrosylation of caspase and possibly regulation of mitochondrial function) (Fiorucci and Del Soldato, 2003). This synergistic activity of the two pharmacophoric moieties, aspirin and NO, potentiates the pharmacological profile of NCX 4016, in comparison with the “parent” drug, and gives to NCX 4016 the *status* of completely new chemical entity.

The effectiveness of NCX 4016 on platelets and on other cellular and metabolic functions relevant to thrombosis has been characterized in *in vitro* studies (Gresele et al., 2003a).

According to these studies, NCX 4016 shows a wider range of action than ASA and inhibits platelet aggregation induced by both aspirin-sensitive and aspirin-insensitive mechanism (Minuz et al., 1995). In fact, NCX 4016 is able to inhibit platelet aggregation induced not only by arachidonic acid and collagen, inhibited also by aspirin, but also the aggregation induced by U46619, a thromboxane analogue, and by thrombin, which are ASA-insensitive (Wallace et al., 1995; Minuz et al., 1995; Fiorucci et al., 2001; Mezzasoma et al., 1999; Lechi et al., 1996; Minuz et al., 2001).

Since also classic NO donors are able to inhibit the platelet aggregation induced by U46619 and thrombin, it seems clear that the NO-moiety of NCX 4016 greatly contributes to the antiplatelet property (Wallace et al., 1995; Minuz et al., 1995; Mezzasoma et al., 1999; Lechi et al., 1996; Minuz et al., 2001; Wallace et al., 2002). Moreover NCX 4016 is able, like SNP but differently from aspirin, to decrease platelet adhesion to collagen under flow conditions and to prevent shear stress-induced platelet activation (Mezzasoma et al., 1999; Negrescu et al., 1995; Ruggeri, 1993). These data are interesting, because adhesion is the first step in the pathway of platelet activation which leads to thrombus formation and only few of the antiplatelet drugs today available are able to prevent it, and a high shear stress like that which occurs in stenosed or atheromatous arteries is retained responsible for the *in vivo* platelet activation.

Remaining in the vascular district, a possible clinical application of NCX 4016 seems to be the prevention of restenosis due to neointimal hyperplasia, which represent the main long-term complication of percutaneous transluminal coronary angioplasty (PTCA), the most frequent treatment to resolve the coronary atherosclerotic stenosis (Cannon et al., 2001). Restenosis is often associated with an impairment of NO-dependent pathways: the modulation of the NO-enzymatic pathway could influence the development of restenosis, i.e., *L*-NAME, (*L*-N_{ω}-nitroarginine methyl ester, an inhibitor of NO synthase) stimulates neointimal hyperplasia. Moreover, NO controls also other pathophysiological responses occurring during atherosclerotic restenosis: inflammatory adhesion, vascular reactivity, endothelial permeability and regulation of smooth muscle cell proliferation linked with the vasculature remodelling. Thereby, as expected, in a model of restenosis in hypercholesterolaemic mice, NCX 4016 has been found to reduce restenosis more efficiently than aspirin and at lower doses (Ignarro et al., 2001; Napoli et al., 2001).

Nitroaspirin could represent also a new approach for the treatment of saphenous vein graft failure which could occur after an autologous saphenous vein coronary artery bypass grafting (CABG) and which are represented by thrombosis, increasing of media thickening and neointima formation, and proliferation and migration of vascular smooth muscle cells (VSMC).

Aspirin affects thrombosis but not the other responses which are instead inhibited by NO, so a NO-releasing aspirin could be a strategy to contrast all these aspects (Shukla et al., 2003).

Finally, an intriguing application for NCX 4016 seems to be represented by a cardioprotective role in myocardial ischemia-reperfusion process. Aspirin is presently used for reducing mortality from acute myocardial infarct but its effects are most likely due to prevention of re-infarction rather than a direct cardioprotective effect, i.e. on arrhythmias (Verheugt et al., 1990).

NCX 4016 reduces infarct size in several animal models but recently its activity has been well characterized in rats. NCX 4016 showed remarkable cardioprotection in rats submitted to myocardial ischemia/reperfusion, as was evident in the reduction of ventricular premature beats and in the incidence of ventricular tachycardia and fibrillation; these arrhythmias were reduced dose-dependently, resulting in survival of all rats treated with higher dose of NCX 4016. Also the infarct size was restricted proportionally to the dose of NCX 4016 and this action was associated with biochemical data such as diminution of both plasma creatine phosphokinase and cardiac myeloperoxidase activities (Rossoni et al., 2001).

An exhaustive overview on the interesting pharmacological properties and on the relatively safe toxicological profile of NCX 4016, emerging from both pre-clinical and the first clinical studies has been recently published (Di Napoli and Papa, 2003). In this article no mention is done about the possible induction of tolerance. However, a bidirectional cross-tolerance between NCX 4016 and glyceryl trinitrate has been described in an *in vitro* study (Grosser et al., 2000).

NO-statins

Inhibitors of 3-hydroxy-3methylglutaryl CoA reductase, also called statins, are the most employed drugs in the treatment of hypercholesterolemia. The efficacy of statins in reducing low-density lipoprotein-cholesterol levels has been confirmed by several clinical trials (Heart Protection Study Collaborative Group, 2002). However, in the last years, the theory of the "pleiotropic effects" of statins is raising. According to this hypothesis, there are other mechanisms of action, beyond lipid-lowering activity, which participate to the beneficial effects of statins in atherosclerotic disease (Bonetti et al., 2003; Marz and Koening, 2003). In fact, statins are able to elicit an increase in endothelial NO production which leads to an anti-inflammatory action at the endothelium level and to an inhibition of VSMC proliferation (Weutz-Schmidt, 2002); but often, especially in those diseases like atherosclerosis and diabetes, there is an impairment of endothelium function which reduces the production of endogenous NO, so NO-releasing statins could offer an alternative source of NO for the therapies of these pathologies.

A recent study on the NO derivatives of pravastatin (NCX 6550) and fluvastatin (NCX 6553) (Fig. 8) shows that the NO-donor moiety significantly potentiates the nonlipid-lowering mechanism of action of native statins.

For example, as concerns the restoration of endothelial function, NO-releasing statins determine a reduction in both smooth muscle cells proliferation and inflammatory events, beyond those induced by statins alone in atherosclerosis (Ongini et al., 2004).

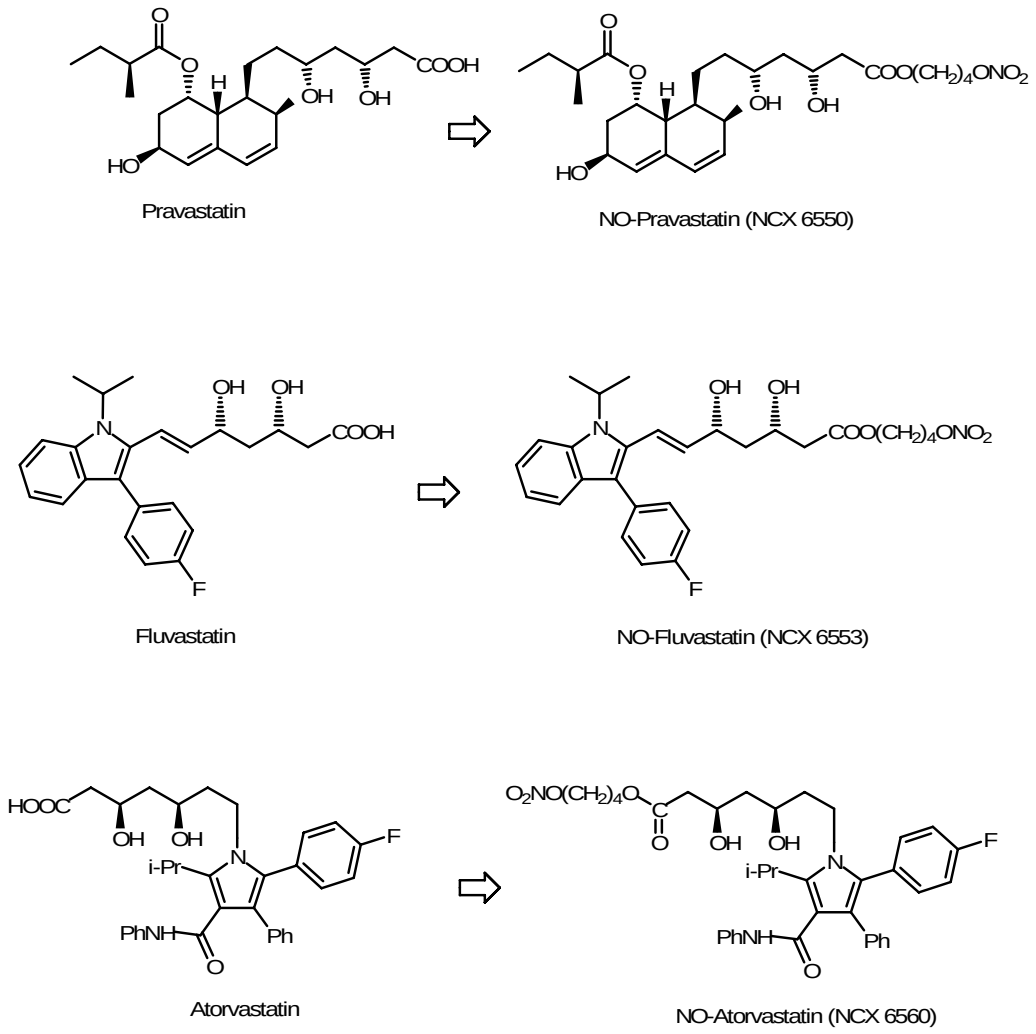


Figure 8. Chemical structures of pravastatin, fluvastatin and atorvastatin, with their corresponding NO-releasing hybrid derivatives.

A chronic treatment with the nitropravastatin derivative, NCX 6550, is able to improve the endothelial dysfunction also in spontaneously hypertensive rats (SHR), a model with marked vascular dysfunction and normal levels of cholesterol (Presotto et al., 2005). Moreover the NO moiety brings additional properties to the native statins, as demonstrated by NCX 6550 which showed the capacity to inhibit platelet aggregation *in vitro,* and to reduce mortality in the

thromboembolism mouse model (Gresele et al., 2003b). More recently the nitropravastatin ability of improve neovascularisation and recovery from limb ischaemia in type-1diabetic mice was also demonstrated; not only but in the same study it was also showed that the *in vitro* pre-incubation with NCX 6550 completely reversed the reduced migratory capacity of endothelial progenitor cells due to high glucose concentration only partially reversed by pravastatin (Emanueli et al., 2007). Finally, the last derivative, the NO-releasing atorvastatin (NCX 6560) (Fig. 7), has been recently compared with atorvastatin in cell-based assays as well as in normal and hypercholesterolaemic mice. These studies show a retained HMG-CoA reductase inhibitory activity but, at the same time, in a model of platelet pulmonary thromboembolism in mice, NCX 6560, significantly reduced mortality, while an equimolar dose of atorvastatin was ineffective. Moreover in LDLR (Low Density Lipoprotein Receptor) knock-out mice fed with hyperlipidemic diet, NCX 6560, but not atorvastatin, significantly reduced *ex vivo* platelet adhesion to collagen under high shear rate (Monopoli et al., 2005). More in particular, recently, it has been observed that, in hyperlipidemic mice, NCX 6560 was more effective than atorvastatin at lowering serum cholesterol; not only but its ability to induce vasodilation, cGMP formation and to reduce epinephrine-induced platelet pulmonary thromboembolism in mice was confirmed. Finally this new molecule seems to exert also an anti-inflammatory activity reducing iNOS expression and TNFα release in LPS (lipopolysaccharide)-trated macrophages (Momi et al., 2007).

NO-Sildenafil

Sildenafil is a selective inhibitor of phosphodiesterase type 5 (PDE5) and it is the pioneer drug in the oral therapy of erectile dysfunction. Its mechanism of action consists in the inhibition of a cGMP phosphodiesterase V subtype, particularly present in penile smooth muscle; in this way, it prevents cGMP hydrolysis, thus allowing a prolonged signalling actions of NO (Corbin and Francio, 1999).

In patients with stable angina, co-administration of sildenafil with organic nitrates produces significant reductions in blood pressure, greater than those induced by nitrates alone. On the basis of these findings, sildenafil should not be associated to a therapy with nitrates because it could lead to an exacerbation of negative and potentially life-threatening cardiovascular consequences (Kloner, 2000).

Although the association of sildenafil with NO-donors seems to be deleterious, in last years a nitroderivative of sildenafil, NCX-911 (Fig. 9), has been synthesised.

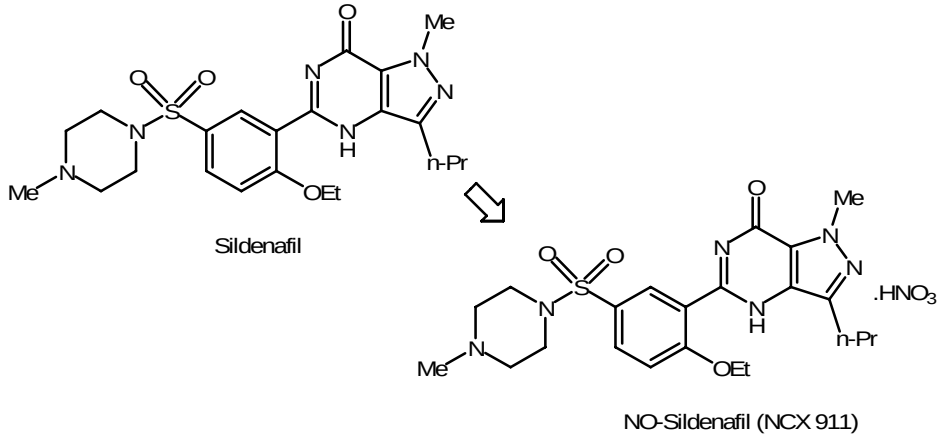


Figure 9. Structures of sildenafil and its nitrate salt derivative, a well-representative example of effective NO-releasing hybrid, based on a simple chemical manipulation.

By a chemical point of view, it could be probably considered as a simplest and effective example of pharmacodynamic hybrid: the NO-donor property has been conferred to the "native" sildenafil through the conversion in its corresponding nitrate salt. A lot of studies were carried out on this new molecule: thus, NCX-911 was found to be as potent as sildenafil in inducing relaxation of rabbit *corpus cavernosum*, but in the presence of *L*-NAME (a NO synthase inhibitor) the potency of NCX-911 was not altered, while sildenafil potency decreased five-fold. This result suggests that NO-releasing PDE5 inhibitors could potentially be more useful than PDE5 inhibitors in the treatment of erectile dysfunction, in conditions of reduced availability of endogenous NO (Kalsi et al., 2004a). For example, in diabetes the endogenous NO is significantly decreased and a comparative study between NCX-911 and sildenafil on the anococcygeus muscle of diabetic rats showed that the potency of NCX-911 in reducing the phenylephrine-induced tone was not altered, while that of sildenafil was significantly reduced (Kalsi et al., 2004b). Another cardiovascular disease, hypercholesterolaemia, promotes erectile dysfunction through increased superoxide formation and negation of NO bioactivity in cavernosal tissue. Although the origin of this mechanism was still not explained, the effect of sildenafil citrate and NCX-911 was studied on cavernosal tissue of hypercholesterolaemic rabbits. The results of this study seem to suggest that NO donating sildenafil may be therapeutically more beneficial than conventional sildenafil in treating erectile dysfunction with an oxidative stress-related aetiology (Shukla et al., 2005).

NO-releasing anti-adrenergic drugs

In the cardiovascular district, the effects of the adrenergic system are mainly mediated by the subtypes 1 and 2 of its α and β receptors. In particular α_1 receptors, when activated, are able to exert vasoconstriction at peripheral level through the transductional system in which IP3 and DAG are involved as second messengers. The use of α_1-antagonist as vasodilators is exercised since several years but, in the last decade, new NO-donor α_1-antagonists were synthesised by replacing the furan ring of prazosin (a well known α_1-blocker) with a furoxan derivative (Fig. 10), to obtain a series of well balanced hybrids in which the vasodilation mediated by α_1-antagonism was integrated with the NO-mediated one (Fruttero et al., 1995). Of course, this strategy led to a structural change of the "native" drug, rather than to a cleavage of the "native" prazosin, following the release of NO.

Nevertheless, among the adrenergic receptors, the principal target for antihypertensive therapy is undoubtedly represented by the β ones. As concerns the cardiovascular system, the β_1 receptors are principally distributed at cardiac level and their activation determine a positive inotropic, chronotropic, batmotropic and dromotropic effect; the β_2 receptors, instead, are responsible of the vasodilation in the skeletal muscle district but their involvement as antihypertensive target is limited. Besides, the β_1 receptors are widely expressed also at the level of renal macula densa, where their block prevents the release of renin and as a consequence, contrasts the formation of angiotensin II through the renin-angiotensin system (RAS). Thereby, selective β_1-blockers exert their antihypertensive effects through several pharmacodynamic patterns, and today

they are commonly used not only to counteract hypertension, thanks to the reduction of cardiac activity and the block in renin release, but also in angina pectoris, thanks to the reduction of cardiac consumption of O_2, and in cardiac arrhythmias.

Recently, in order to potentiate some β_1-blockers activities, such as the lowering effects on the blood pressure and the decreasing in oxygen consumption, several hybrid molecules were synthesised, in which different NO-moieties (furoxanes, 3-nitrooxypivaloyl acid, *N*-acetyl-$_D$-penicillamine) were linked to chemical structures, closely related with propranolol (Fig. 10) (Boschi et al., 1997; Decker et al., 2004).

Some of these new hybrid molecules were well-balanced and exert both the β-blocking and the NO-releasing actions in the same range of concentration, but in general, compared with propranolol, the hybrid formation lowers the affinity for β-receptors, in particular for the β_2-type, to give an increase in β_1/ β_2 selectivity (Boschi et al., 1997).

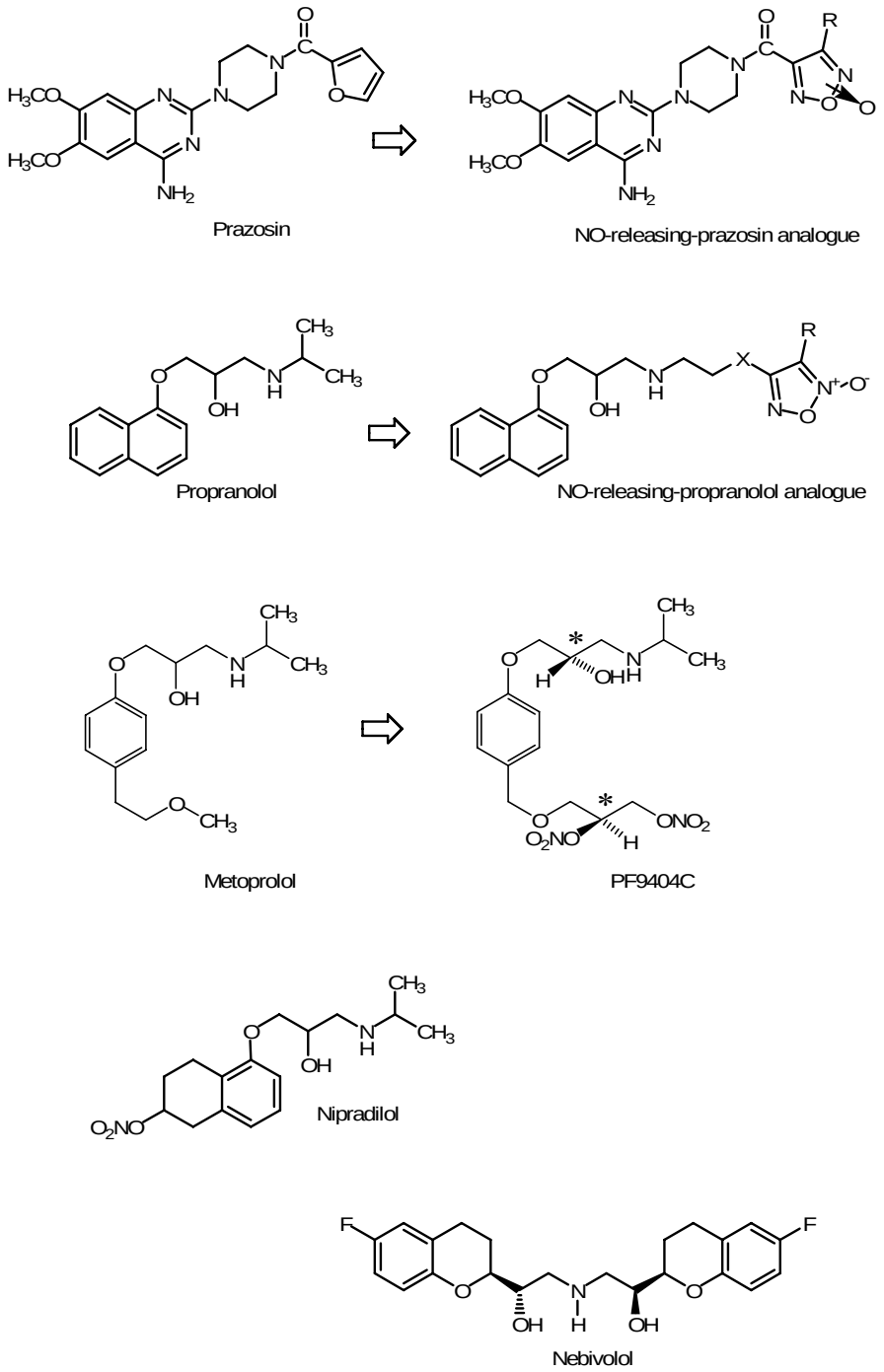


Figure 10. Chemical structures of prazosin (α1-blocker) propranolol and metoprolol (β-blockers) and their NO-releasing analogues. When the NO molecules are metabolised, lead to new compounds. Nipradilol possesses a nitrooxy group, conferring NO-releasing properties. Nebivolol does not possess any NO-releasing moiety, nevertheless it is able to induce an increased release of endothelial NO and thus, nebivolol shows pharmacological effects which can be assimilated to those produced by a hybrid NO-β-blocker.

An interesting new hybrid β-blocker is represented by a S-S enantiomer of a metoprolol-related derivative, PF9404C, studied and developed in order to correct the initial high peripheral resistance occourring in hypertensive patients treated with β-blockers. On the other hand, due the β-blocker activity, it could prevent the increase of heart rate and catecholamine levels which represent the reaction to a fall of peripheral resistance and blood pressure.

In a previous study PF9404C was compared with rapid NO-donors producing an immediate but transient vasorelaxation and what was new is that, on the contrary, it seems able to evoke a slowly developed but sustained relaxation (Villaroya et al., 1999).

Moreover, although classic nitrovasodilators were often associated with β-blockers as an efficient therapeutic approach in coronary heart disease, the phenomenon of tolerance, rising in chronic treatment, could not be ignored. A recent study demonstrates that if compared with nitroglycerin, PF9404C shows much lesser tolerance. This lower induction of tolerance by PF9404C could be explained through its slower release of NO (Ruíz-Nuño et al., 2004).

Nipradilol (3,4-dihydro-8-(2-hydroxy-3-isopropyl-amino)-propoxy-3-nitroxy-2H-1-benzopyran) (Fig. 9) is a nonselective adrenoceptor blocking agent with a weak β-adrenoreceptor blocking activity and direct vasodilating property due to a nitro group contained within its molecular structure (Uchida et al., 1983). Nipradilol showed beneficial effects on cardiac remodelling, ischemic heart diseases and blood pressure control (Kosegawa et al., 1998; Sonoki et al., 1997), and its

involvement on both intracellular calcium mobilization and the content of cGMP was proposed to partially explain its pharmacological action (Nitta et al., 1998). In a recent study it was hypothesised that mechanical stress-induced activation of intracellular signal transduction cascades is controlled by nipradilol in human aortic smooth muscle cells (HASMC) (Iizuka et al., 2004). In fact, in endothelial cells, mechanical stresses are able to stimulate one or more phosphorylation cascades leading to an activation of mitogen-activated protein kinases (MAPKs) (Tseng et al., 1995). Also hypertension and angioplasty are rapid inducers of MAPKs activation *in vivo* (Pyles et al., 1997). These findings confirm that nipradilol has an action in modulating intracellular signal transduction pathway, i.e. extracellular signal-regulated kinase (ERK) cascade under high atmospheric pressure on HASMC (Iizuka et al., 2004), but classical β-blockers are reported to lack an inhibitory effect on SMC proliferation (Brehm et al., 2001). Therefore, the anti-proliferative effect of nipradilol on HASMC was thought to be caused by the release of NO itself (Iizuka et al., 2004). Different studies suggest that nipradilol is a potent NOS stimulator in endothelial cells with NO releasing action, so this drug could represent a substitute for injured endothelial cells with a possible role in the treatment of endothelial dysfunctions occurring in atherosclerosis, hyperlipidemia and hypertension (Zeiher et al., 1993; Jaychandran et al., 2001).

Finally, nipradilol mimics a cardioprotective mechanism called preconditioning. Preconditioning represents a protective system in which a brief period of ischemia, preceding a most important ischemia, determines a markedly reduced infarct size. This physiological endogenous mechanism can be mimicked through the administration of some exogenous drugs, such as ATP-sensitive potassium-

channel openers, adenosine, etc. (Parrat and Kane, 1994; Yao and Gross, 1993; Yokota et al., 1995). Nitric oxide is retained to be involved in the mechanism of preconditioning (Vegh et al., 1992), and recent studies have shown that NO is a requisite for cofactor in the preconditioning response generated by the administration of ATP-sensitive potassium-channel openers (Horimoto et al., 1998). After a coronary surgical intervention a lot of patients receive β-blockers, but β-adrenoceptors blockade alone prevents preconditioning, so the proposal to administer nipradilol may simultaneously offer a β-blocking action and a NO-mediated preconditioning. As concerns mechanical function, nipradilol may exert its effect by increasing coronary flow after the reperfusion (Horimoto et al., 1999).

Although nebivolol is not a molecule able to release exogeous NO (Fig. 9), it is able to induce an increased release of endothelial NO (as better explained below). Even if it can not be considered as a true "bifunctional" drug, nevertheless it shows pharmacological effects which can be assimilated to those produced by a hybrid NO-β-blocker. Nebivolol is a racemic mixture of equal amounts of the two enantiomers *D* an *L*, which belongs to the class of the high cardioselective, non-hybrid, β-adrenergic receptor antagonists. Its peculiarity is that nebivolol possesses vasodilator properties, which are not due to the block of adrenergic receptors on smooth muscle cells. This vasodilator response is attributed to *L*-nebivolol and experimental evidence indicates that it is due to endothelium-dependent mechanism involving NO and the *L*-arginine/NO pathway (Van de Water et al., 1988; Gao et al., 1991; Cockroft et al., 1995). Recent *in vitro* findings show that nebivolol relaxes vascular smooth muscle by mechanism

involving the NO-cyclic GMP system (Ignarro et al., 2002b); these observations are predictive of *in vivo* effects in patients and support the findings that nebivolol reverses endothelial dysfunction in patients with essential hypertension (Tzemos et al., 2001). Finally, nebivolol is also able to inhibit vascular smooth muscle cell proliferation by mechanisms involving NO but not cyclic GMP (Ignarro et al., 2002c).

NO-releasing dihydropyridines

The 1,4-dihydropyridine (DHP) Ca^{2+}-antagonists, such as nifedipine, nitrendipine, amlodipine, are widely used in the treatment of hypertension and ischemic heart diseases. Their principal mechanism of action consists in the inhibition of Ca^{2+}-influx through voltage-dependent L-type calcium channels (VDCCs) in vascular smooth muscle (Godfraind, 1994; Kuriyama et al., 1995). Besides the effects on the vascular smooth muscle, there are studies which reported that amlodipine may release nitric oxide from canine coronary microvessels through modulating the actions or formation of kinins; however, the mechanism by which calcium antagonists release NO is still unclear because there is no known receptor for Ca^{2+}-antagonists in endothelial cell (ECs) (Zhang and Hintze, 1998) and most ECs lack VDCCs, so endothelial depolarization fails to increase the intracellular Ca^{2+} concentration through VDCCs (Nilius et al., 1997). Recently, it has been found that vascular smooth muscle and endothelial cells are tightly coupled via myo-endothelial communication and that electrical signals can be conducted each other (Yamamoto et al., 1999; Murai et al., 1999). This finding suggests that the

modulation of electrical signals in smooth muscle by DHPs could affects membrane potential in ECs (Muraki et al., 2000).

In order to potentiate their vasorelaxing properties, DHP Ca^{2+}-antagonists were structurally modified by the addition of furoxan moieties which act as NO-donors. So a new series of 4-phenyl-1,4-dihydropyridines, bearing furoxan moieties at the *ortho* or *meta* position of the phenyl ring was synthesized (Fig. 11) and pharmacologically characterised, allowing to distinguish the well balanced hybrid molecules from that having a Ca^{2+}-antagonist or NO-donor dominant profile (Di Stilo et al., 1998).

Finally, in the last years, also the properties of NO-donor Ca^{2+}-agonists were investigated: in fact, when an appropriate group, such as the nitro group, is inserted in one of the two ester functions of the 1,4-dihydropyridine structure, the resulting two enantiomers display opposite pharmacological profile as in Bay K 8644, in which the (-)-*S*-antipode is a potent agonist at L-type Ca^{2+}-channels while the (+)-*R*-antipode is a weak antagonist (Goldmann and Stoltefuss, 1991). Ca^{2+}-agonists are positive inotropic agents so they are potentially useful for the treatment of the congestive heart failure (CHF), but their capacity to increase Ca^{2+} levels in vascular smooth muscle represent a limit because it leads to vasoconstriction. The combination of these 1,4-DHPs able to activate L-type Ca^{2+}channels with a NO-donor moiety (2-nitrooxyethyl esters, furoxanes or diazeniumdiolates) (Fig. 9) could represent a strategy to overcome this adverse effect and therefore to realize a new class of hybrid compounds which show a positive inotropism devoid of vasoconstriction (Visentin et al., 2004; Velázquez and Knaus, 2004); Shan and Knaus, 1999; Shan et al., 2002).

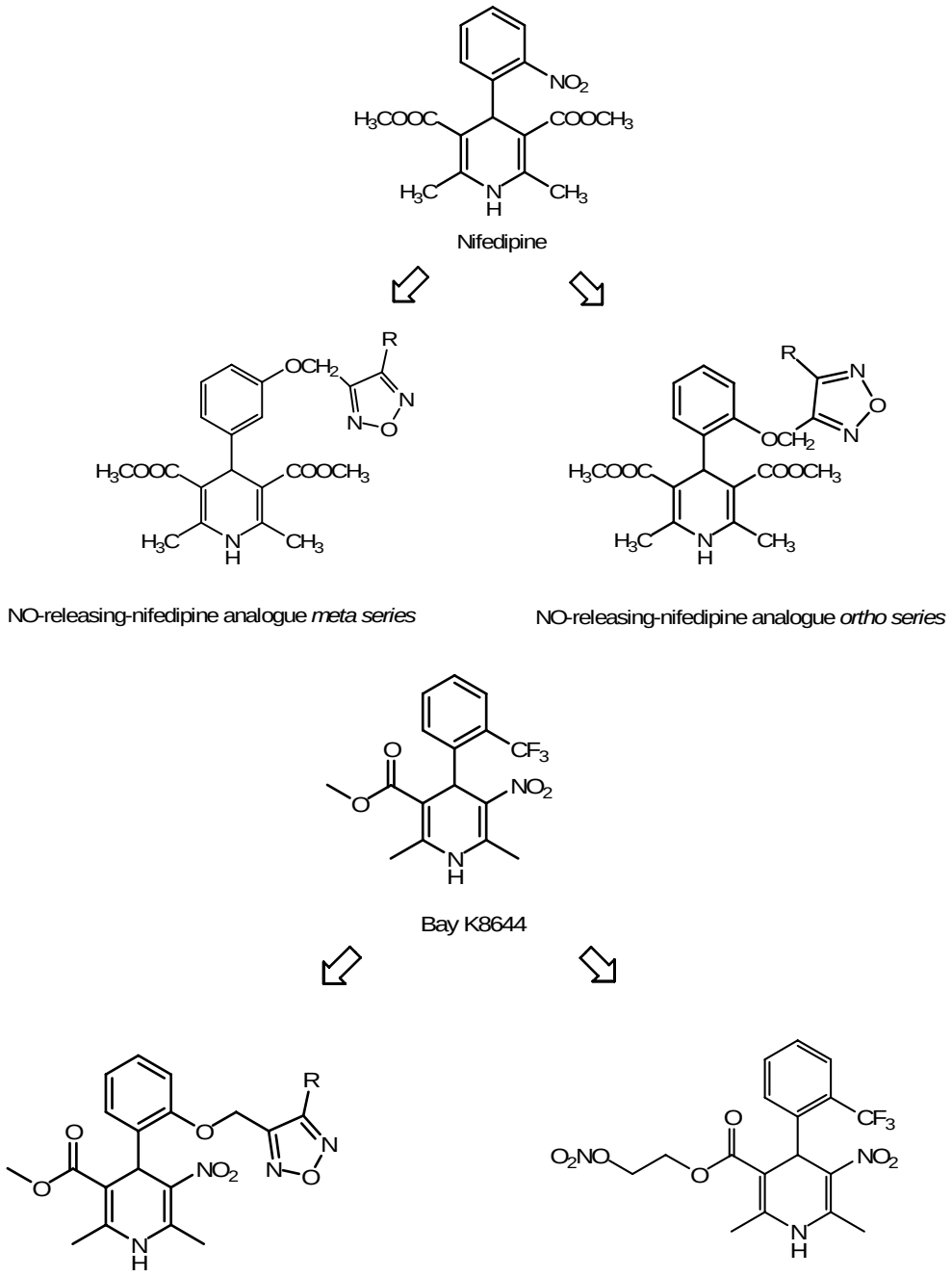
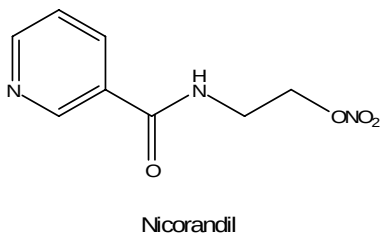


Figure 11. Chemical structures of the dihydropyridines nifedipine and Bay K8644 (respectively, calcium antagonist and calcium-agonist), and of their NO-releasing analogues. When the NO-dihydropyridines are metabolised, lead to new compounds.

Nicorandil

Nicorandil, *N*-(2-hydroxyethyl)-nicotinamide nitrate ester (Fig. 12), is an hybrid anti-anginal drug that possesses the characteristics of both an ATP-sensitive potassium channel (K_{ATP}) opener and a NO-donor.



Nicorandil

Figure 12. Chemical structure of nicorandil. This K_{ATP} –activator shows a nitrooxy group, conferring the NO-donor property, as an additive pharmacodynamic feature.

Nicorandil exerts its vasodilating effect through a dual mechanism of action: as a nitrovasodilator it activates the guanylate cyclase increasing the cGMP formation and these events lead to a relaxation of vascular smooth muscle. As a K_{ATP} opener nicorandil determines the hyperpolarization of the surface membrane which causes the closure of voltage-dependent ion channels and the reduction of free intracellular calcium ions and even this pathway leads to a vasodilatory effect. Due to its K_{ATP} opener profile, nicorandil dilates peripheral and coronary resistence arterioles while the nitrate moiety allows it to dilate systemic veins and epicardial coronary arteries. So nicorandil increases coronary blood flow, reduces preload and afterload (Taira, 1987; Yokota et al., 1987) and exerts an anti-anginal effect comparable with the nitroglycerin one (O'Rourke, 1996; Satoh et al., 1993). However nitrate therapy might induce the development of tolerance, increase vascular sensitivity to vasoconstrictors (Elkyan, 1991) and deteriorate endothelial function (Caramori et al., 1998). Indeed nicorandil, due to its dual mechanism of

action, is able to determine vasorelaxation even in nitrate-tolerant blood vessel through the opening of K_{ATP} channel (IONA, 2002).

Moreover the clinical trial "Impact of Nicorandil in Angina" (IONA study) showed that nicorandil improves the prognosis of patients with stable angina pectoris by reducing the frequency of acute coronary syndrome (ACS) (IONA, 2002). Recent studies seem to suggest that this reduction is possible because nicorandil may be able to inhibit intracoronary thrombus formation through modification of the type-1 plasminogen activator inhibitor (PAI-1) (Sakamoto et al., 2004). In last years, several studies demonstrated that the activation of mitochondrial K_{ATP} (mito K_{ATP}) channel by diazoxide is able to inhibit apoptosis induced by oxidative stress in cardiac myocytes; the same studies found that also nicorandil exerts an anti-apoptotic action through the activation of mitoK_{ATP} (Akao et al., 2001; Akao et al., 2002). More recent experimental findings, obtained through the coapplication of the mitoK_{ATP} channel antagonist 5-hydroxydecanoate (5-HD) and of the inhibitor of soluble guanylate cyclase ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), on cultured myocytes treated with hydrogen peroxide and nicorandil, seems to indicate that also the nitrate-like effect contributes to the inhibition by nicorandil of apoptosis induced by oxidative stress in cardiac myocytes (Nagata et al., 2003).

Finally, nicorandil is known as a cardioprotective agent acting in the ischemic preconditioning through the opening of mitoK_{ATP} channels, however the exact mechanism of action is still unclear, probably involving several components (Sato et al., 2000; Iwai et al., 2002). For example, a key role seems to emerge for NO which, according to recent data, selectively activates mitoK_{ATP} channels through

the protein kinase C (PKC) translocation from the cytosolic to the mitochondrial fraction (Harada et al., 2004). The pivotal role of PKC, in cardioprotection upon ischemia-reperfusion has been reported (Kawamura et al., 1998; Liu et al., 1994; Mitchell et al., 1995). Therefore, probably nicorandil exerts its anti-ischemic effect through synergistic mitoK_{ATP} channel opening generated by a direct effect, together with NO-donor activation of PKC; this dual mechanism of action could explain the greater effectiveness of nicorandil in comparison with other pure K_{ATP} channel openers (Harada et al., 2004).

NO-ACE inhibitors

ACE inhibitors, such as captopril, enalapril etc. represent a first-choice class of drugs in the pharmacotherapy of hypertension and congestive heart failure: they act on renin angiotensin system (RAS) preventing the conversion of angiotensin I to angiotensin II (AII) by the Angiotensin Converting Enzyme (ACE). In last years, in order to potentiate the ACE inhibitors properties, a *S*-nitrosylated derivative of captopril, S-nitrosocaptopril (SNOcap) (Fig. 13), was synthetized and characterized (Jia and Blantz, 1998). Its NO-donor and ACE inhibiting actions emerge after the homolytic cleavage of the S-NO bond, under physiological conditions (Jia et al., 1999). The pharmacological properties of SNOcap have been described in systemic vessels, *in vitro* (Loscalzo et al., 1989; Cooke et al., 1989) and *in vivo* (Shaffer et al., 1991; Nakae et al., 1995). Due to the dual mechanism of action, S-nitrosocaptopril is effective in hypertension, platelet aggregation, congestive heart failure and pulmonary hypertension,

potentiating the effect of the native captopril (Tsui et al., 2003). This improved mechanism of action is due not only to the sum of the two components.

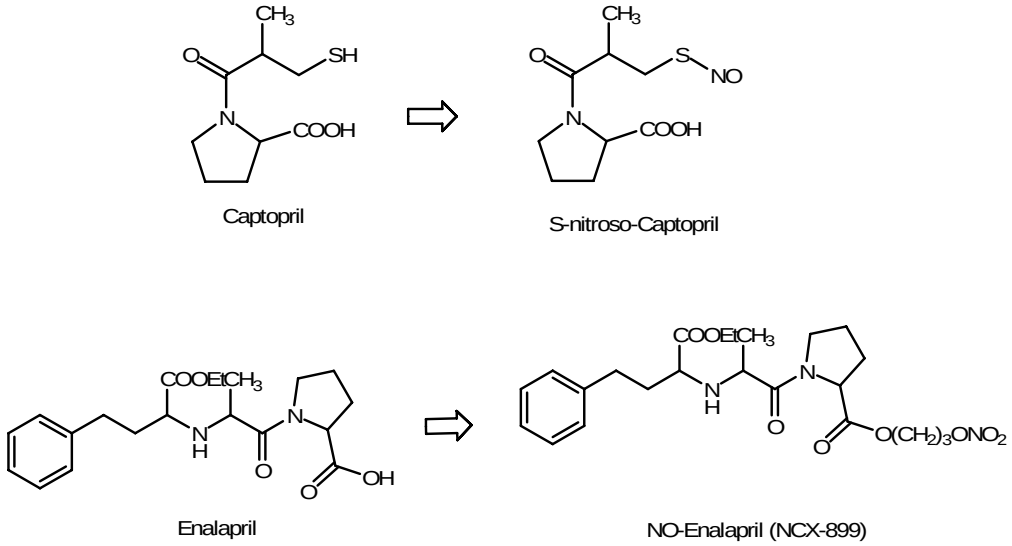


Figure 13. Chemical structures of the ACE-inhibitor drugs captopril and enalapril and of their NO-releasing hybrids. In such hybrids, the removal of the NO-donor moieties leads to the original "native drugs, without any structural alteration.

Recently, Ackermann et al. showed that NO and NO-releasing substances can competitively inhibit ACE (Ackermann et al., 1998) and this finding was confirmed by other studies on porcine iliac arteries (Persson and Andersson, 1999) and on rats pulmonary artery (Tsui et al., 2003), comparing SNOcap with the parent drug, captopril. Finally, the toxicity of SNOcap was examined in rodents: the absence of adverse effects in subchronic toxicity studies makes SNOcap a good candidate for further clinical trials, but it could cause a severe hypotension when overdosed (Jia et al., 2001).

More recently, a new compound, NCX 899, a NO-releasing derivative of enalapril (Fig. 13) was synthetized and characterized, evaluating its action on cardiomyopathic hamsters with heart failure. NCX 899 is a slow NO-donor and the cleavage of the native molecule from the NO-moiety is due to the action of

esterases. If compared with enalapril alone, NCX 899, appears more effective in enhancing vascular effects, increasing left ventricular contractility and preventing unfavourable remodeling, consistently with a vascular delivery of exogenous NO which determines an improvement in endothelial function and a reduction of vascular resistence (Iwanaga et al., 2004). Moreover, in a recent study, the pharmacokinetics and pharmacodynamics of NCX899 was evaluated in male beagles: the results showed that NCX899 presented pharmacokinetic characteristics similar to the native drug enalapril and it maintained the ACE-inhibiting profile but it was also effective in protecting against the raise of arterial blood pressure and the concomitant bradycardia induced by an i.v. administration of the NO-synthase inhibitor, L-NAME (Okuyama et al., 2007).

CHAPTER 2

NO-DONOR LINKERS: EVALUATION OF THEIR DIFFERENT NO-RELEASING PROPERTIES[*]

[*] The contents of chapter 2 have been already published:

Calderone V., Digiacomo M., Martelli A., Minutolo F., Rapposelli S., Testai L., Balsamo A.

J. Pharm. Pharmacol., in press

Introduction

In order to obtain a multi-target drug possessing NO-donor properties, it was important to conjugate the NO function to another drug with an easily cleavable group. The ester-based linker is a cleavable group which, as a result of the action of plasma esterases, is capable of releasing two individual drugs (nitric oxide and the "native drug"), which act independently.

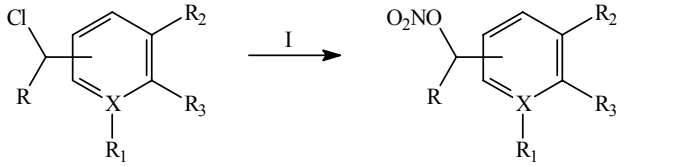
In the last few years, many cleavable conjugates have been described (see Introduction) containing an NO-releasing functionality linked via an ester group to a known "native" drug.

The chemical strategies usually employed to add NO-donor properties to a "native" drug involved its conjugation with different nitrogen-containing molecular portions. Among them, the nitrooxy moiety, conjugated to a "native" drug often through a molecular linker, probably represented the most convenient approach.In order to ensure an appropriate conjugation between the NO-donor linker and a potential native drug, the nitrooxy group was inserted into different structures possessing carboxylic, phenolic or alcoholic functions suitable to form a vulnerable bond such as the ester one. On this basis, with the aim of study the pharmacologic properties of these NO-donor structures potentially useful to build up new pharmacodynamic hybrids, the nitrooxymethyl-benzoic acids (**Ar-NO** [**1a**]; **p-Ar-NO** [**1b**]; **Ar-NO-α-Me** [**2a**]; **p-Ar-NO-α-Me** [**2b**]; **Ar-NO-2,6-Me_2** [**3**]) and the 2-nitrooxymethyl-pyridinecarboxylic acid (**Pyr-NO** [**4**]) have been synthesised; furthermore, as hydroxy-functionalised linkers the phenol derivative (**Phen-NO** [**5**]) and four nitrooxymethyl-benzylic alcohols (**Benz-NO** [**6a**]; **p-Benz-NO** [**6b**]; **Benz-NO-α-Me** [**7a**]; **p-Benz-NO-α-Me** [**7b**]) were also

synthesised (Scheme 1,2). In Scheme 1 is represented the general procedures following to obtain nitrooxy derivatives. As concern compounds **Ar-NO, p-Ar-NO, Ar-NO-2,6-Me$_2$, Phen-NO, Benz-NO** and **p-Benz-NO** they were obtained by reaction of the corresponding chloro-derivatives with silver nitrate in acetonitrile (method A, sch. 1), while compounds **Ar-NO-α-Me, p-Ar-NO-α-Me** and **Pyr-NO** were obtained by reaction with nitric acid and acetic anidride at –10 °C (method B, sch. 1). As concern compounds **Benz-NO-α-Me, p-Benz-NO-α-Me**, these were synthesised starting from the appropriate acetylbenzoic acids (**8a,b**), which were reduced to the corresponding benzyl-alcohols **9a,b** with $LiAlH_4$ in THF (Scheme 2). The treatment of a solution of **9a,b** in toluene with concentrated HCl afforded the monochloro derivatives **10a,b** which were submitted to reaction with $AgNO_3$ in CH_3CN to yield the corresponding nitro esters **7a,b** (Scheme 2).

Scheme 1.

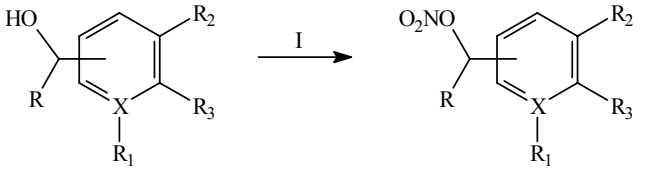
Method A



1, 3, 5, 6

I: $AgNO_3$, CH_3CN, r.t.

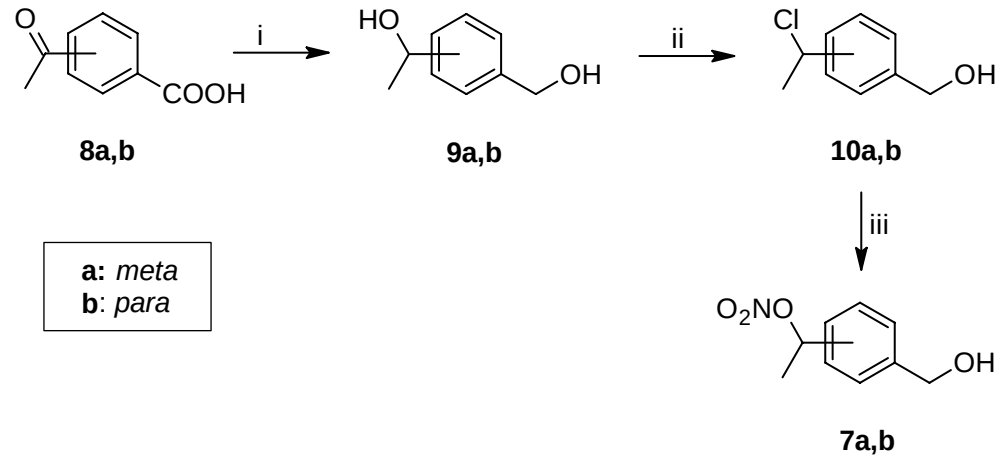
Method B



2, 4

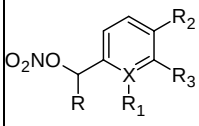
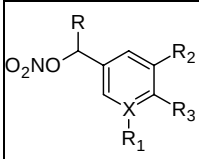
I: $HNO_{3\ conc.}$, Ac_2O, r.t..

Scheme 2.



i: $LiAlH_4$, THF, r.t.; **ii**: $HCl_{conc.}$, toluene, r.t., **iii:** $AgNO_3$, CH_3CN, r.t.

Table 1. NO-donor linkers with a carboxy (**1a,b-4**), phenolic (**5**) or hydroxymethyl function (**6a,b-7a,b**).

	Compound	X	R	R1	R2	R3
	**Ar-NO (1a)**	C	H	H	H	COOH
	Ar-NO-α-Me (2a)	C	Me	H	H	COOH
	Ar-NO-2,6-Me_2 (3)	C	H	Me	Me	COOH
	Pyr-NO (4)	N	H	-	H	COOH
	Phen-NO (5)	C	H	H	H	OH
	Benz-NO (6a)	C	H	H	H	CH_2OH
	Benz-NO-α-Me (7a)	C	Me	H	H	CH_2OH
	**p-Ar-NO (1b)**	C	H	H	H	COOH
	p-Ar-NO-α-Me (2b)	C	Me	H	H	COOH
	p-Benz-NO (6b)	C	H	H	H	CH_2OH
	p-Benz-NO-α-Me (7b)	C	Me	H	H	CH_2OH

Pharmacological procedures

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. The experimental protocol was approved by the Animal Care Committee of the University of Pisa.

The compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250-350 g).

After a light ether anaesthesia, the rats were sacrificed by cervical dislocation and bleeding.

The aortas were immediately excised and freed from extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five millimeter wide aortic rings were suspended, under a preload of 2g, in 20mL organ baths, containing Tyrode solution (composition in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37°C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected to a preamplifier (Buxco Electronics) and to data acquisition software (BIOPAC Systems Inc., MP 100).

After an equilibration period of 60 minutes, endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted rings. A relaxation < 10% of the KCl-induced contraction was considered to be indicative of an acceptable lack of the endothelial layer, while organs showing a relaxation ≥ 10% (i.e., a significant presence of the endothelium) were discarded.

NO-mediated vasorelaxing effect: concentration-response curves

From 30 to 40 minutes after the confirmation of endothelium removal, aortic preparations were contracted by a single concentration of KCl (30mM), and when the contraction reached a stable plateau, 3-fold increasing concentrations of the test substances (from 1 nM to 100 μM) were added.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 minutes.

The same experiments were carried out in the presence of a well-known guanylate cyclase inhibitor, ODQ (1 μM), which was incubated in aortic preparations after confirmation of endothelium removal.

Time-course of vasorelaxing effect

From 30 to 40 minutes after confirmation of endothelium removal, aortic preparations were contracted with a single concentration of 30mM KCl, and after the reaching of a stable plateau a single concentration (1 μM) of the compounds was added. The vasorelaxing effects of the added compounds was monitored for 50 minutes.

Data analysis

The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30mM KCl. When the limit concentration of 100 μM (the highest concentration that could be administered) of the test compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30mM KCl, evoked by this limit

concentration. The parameter of potency was expressed as pIC_{50}, calculated as the negative logarithm of the molar concentration of the test compounds evoking a 50% reduction of the contractile tone induced by 30mM KCl. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50 %. The above experimental data were obtained by a computer fitting procedure from the concentration-response curves (software: GraphPad Prism 4.0).

In order to describe the time-course profiles of the effects, the "effect *vs* time" curves were analysed by a computer fitting procedure (software: GraphPad Prism 4.0), using the following equation:

Eff = (EM x T) / (Kt + T)

where :

Eff is the vasorelaxing effect (expressed as a percentage of the contractile tone induced by KCl 30 mM) recorded at each min, after the administration of the test compounds (1 μM).

EM is the highest vasorelaxing effect (expressed as a percentage of the contractile tone induced by KCl 30 mM) induced by the test compounds (1 μM).

T is the time, expressed in min, correlated to a given Eff.

Kt is the time (in min) required to reach an Eff = ½ EM

This analysis allowed us to calculate the parameter of T25 (time, in min, required to reach an equi-effective level of vasorelaxing effect = 25% of the contractile tone induced by KCl 30 mM).

The parameter of T25 could not be calculated for those compounds exhibiting an EM lower than or close to 25.

The parameters of pIC50 and T25 were expressed as means ± standard error, for 5-10 experiments and were statistically analysed through one-way ANOVA, followed by the Bonferroni post-test. The concentration-response curves and the time-course curves were statistically evaluated through two-way ANOVA.

A level of $P < 0.05$ was considered to be indicative of a significant statistical difference.

Results

The insertion of the nitrooxymethyl chain in the *meta* position (as in compounds **Ar-NO**, **Ar-NO-α-Me, Benz-NO, Benz-NO-α-Me**) or in the *para* position (as in compounds **p-Ar-NO, p-Ar-NO-α-Me, p-Benz-NO and p-Benz-NO-α-Me**) with respect to the carboxylic- (in the benzoic series) or to the hydroxymethyl- function (in the benzylic series) was selected in order to investigate the potential influence played by steric and/or electronic factors on the NO-releasing properties of these compounds.

Moreover the insertion into the aromatic ring (**Ar-NO-2,6-Me$_2$**) or into the carbon atom of the nitrooxymethyl chain (**Ar-NO-α-Me, p-Ar-NO-α-Me** and **Benz-NO-α-Me, p-Benz-NO-α-Me**) of one (or more) methyl group(s) was also affected with the aim of determining the potential influence of these groups on the NO-releasing kinetics of **Ar-NO-α-Me, p-Ar-NO-α-Me**, **Ar-NO-2,6-Me$_2$**, **Benz-NO-α-Me, p-Benz-NO-α-Me.** All the compounds synthesised were tested as vasodilator agents on rat aortic rings pre-contracted with a depolarising stimulus (KCl 30mM). In this experimental model, all compounds exhibited full or almost full vasorelaxing activity. The potency results (expressed as pIC50) and the

efficacy (Emax%) of the compounds tested and of reference drug SNP are given in Table 2, while the parameters describing the time-course profile of the vasorelaxing effect (T25 values), reflecting the NO-release rates, are shown in Table 3. As regards the potency, the pIC50 values were always lower than that exhibited by the reference drug sodium nitroprusside (SNP, pIC50 = 8.73), and ranged between the lowest pIC50 of 4.83 (recorded for compound **Benz-NO-α-Me**) and the highest one of 7.21 (observed for **p-Benz-NO**). The fact that the vasorelaxing effects of all the test compounds and of SNP were almost completely abolished by pre-incubation with ODQ (an inhibitor of guanylate cyclase and thus of the NO-cGMP pathway), indicated a clear involvement of NO-release in their mechanism of action (Fig. 14). Although the potency value in itself was clearly influenced by the rate of NO-release, an analysis more closely focused on the evaluation of the development of the vasorelaxing effect vs time (through the time-course protocol and the T25 parameter) seemed to be a more appropriate approach to describe this aspect. Indeed, the T25 parameter reflects the time needed to achieve a selected level of vasorelaxing effect (25% of the pre-contraction by KCl 30 mM) induced by 1 μM of the compounds tested. Thus, the T25 parameter could be viewed as an indirect indicator of the rate of release of NO from the drug. As expected, SNP (a well- known rapid NO-releasing agent) exhibited a very low T25 value (0.39 min). Two compounds (**Benz-NO** and **p-Benz-NO)** showed T25 values (0.48 min and 0.45 min, respectively) almost the same as to that of SNP. Compound **Phen-NO** exhibited a T25 value slightly higher (2.00 min) than that of SNP, but the two values did not result statistically different. All the other compounds proved to be NO-donor agents, possessing a

significantly lower rate of NO-release, with T25 values ranging between 3.84 min (**p-Ar-NO**) and 8.83 min (**Ar-NO-α-Me**). 1 μM of **p-Benz-NO-α-Me** determined a modest vasorelaxing effect (EM = 26 %), which did not allow the calculation of T25. 1 μM of **Ar-NO-2,6-Me$_2$** and **Benz-NO-α-Me** did not evoke any significant vasorelaxing effect, indicating that these two compounds were the slowest NO-donors of the series (Fig. 15).

Table 2. The NO-mediated vasorelaxing efficacy of the synthesised compounds and SNP was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the test compounds, evoking 50% reduction of the contractile tone induced by 30 mM KCl. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means ± standard error, for 5-10 experiments.

Compound	pIC_{50}	Emax %
SNP	8.73 ± 0.04	100[a]
1a	5.80 ± 0.08	78 ± 9
1b	6.47 ± 0.02	100[a]
2a	5.64 ± 0.04	96 ± 2
2b	5.71 ± 0.02	100[a]
3	5.30 ± 0.03	98 ± 2
4	6.34 ± 0.02	100[a]
5	6.26 ± 0.05	100[a]
6a	7.03 ± 0.04	100[a]
6b	7.21 ± 0.03	100[a]
7a	4.83 ± 0.04	91 ± 1
7b	5.98 ± 0.02	100[a]

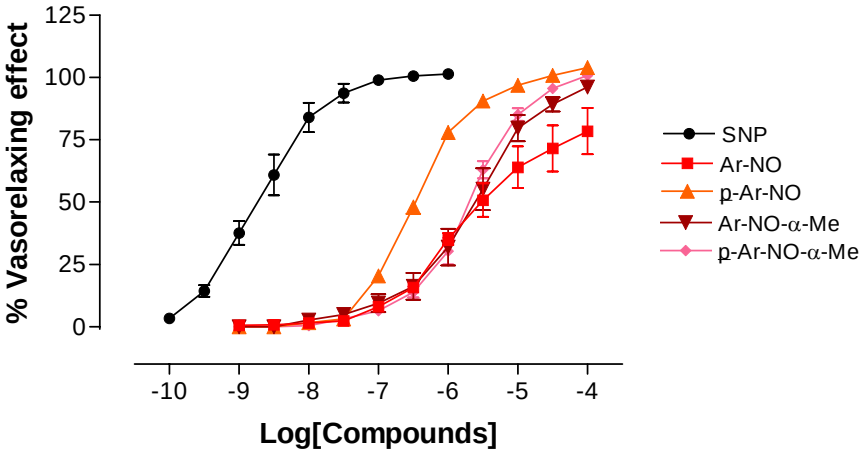
Table 3. The time-course profiles of the effects of the synthesised compounds and SNP is summarised in the table. EM is the highest vasorelaxing effect (expressed as a % of the contractile tone induced by KCl 30 mM) induced by the tested compounds (1 μM). T25 is the time, in min, required to reach a level of vasorelaxing effect = 25% of the contractile tone induced by KCl 30 mM. The parameter of T25 could not be calculated for those compounds, exhibiting an EM lower than 25. Some compounds (shown as ineffective) did not exhibit any significant vasorelaxing effect, at the concentration 1 μM.

The values are expressed as means ± standard error, for 5-10 experiments.

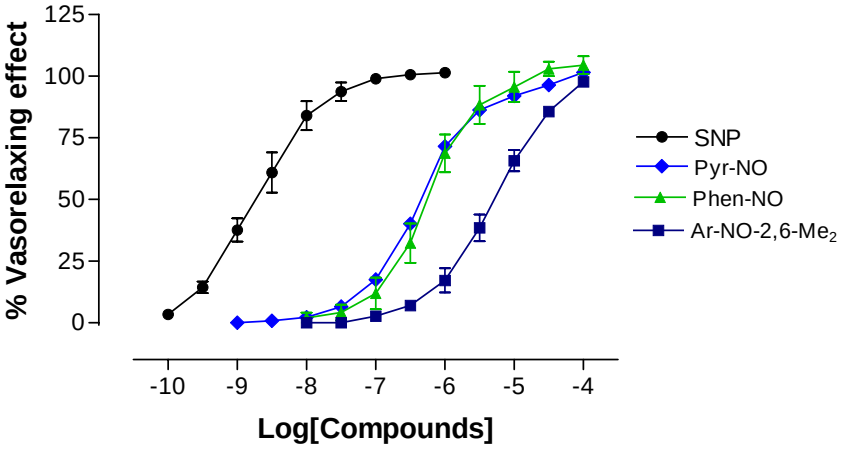
Compound	EM	T25
SNP	98 ± 2	0.39 ± 0.08
1a	39 ± 8	8.25 ± 0.42
1b	65 ± 10	3.84 ± 0.48
2a	42 ± 2	8.83 ± 0.58
2b	50 ± 7	6.25 ± 0.21
3	Ineffective	-
4	49 ± 3	4.68 ± 0.33
5	73 ± 1	2.00 ± 0.27
6a	90 ± 1	0.48 ± 0.02
6b	90 ± 4	0.45 ± 0.03
7a	Ineffective	-
7b	26 ± 1	Not Calculable

Figure 14. The NO-mediated vasorelaxing efficacy of the synthesised compounds and SNP evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl

A



B



C

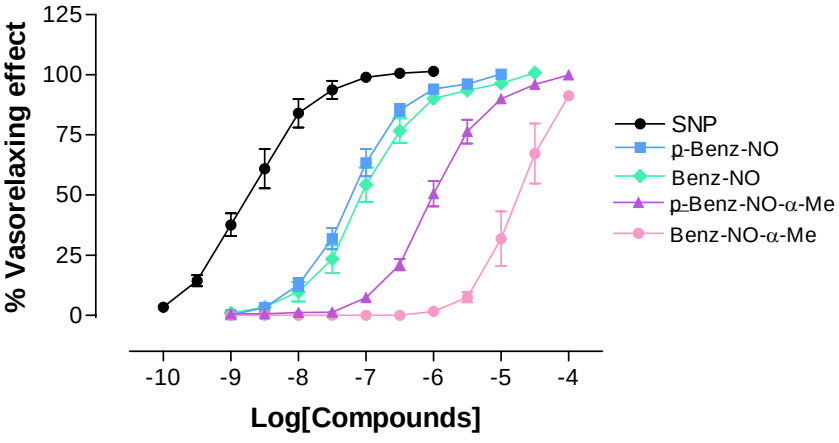
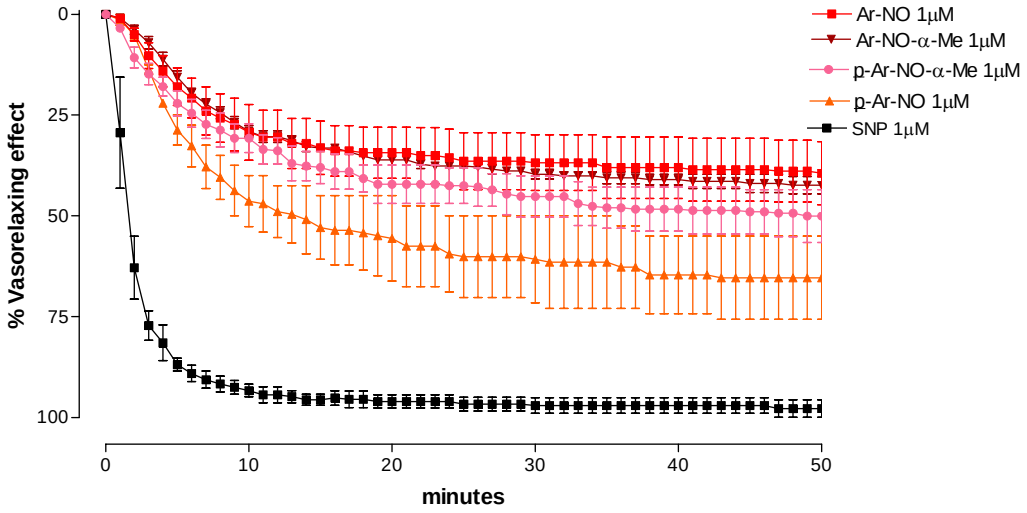


Figure 15. The time-course profiles of the effects of the synthesised compounds and SNP.

A



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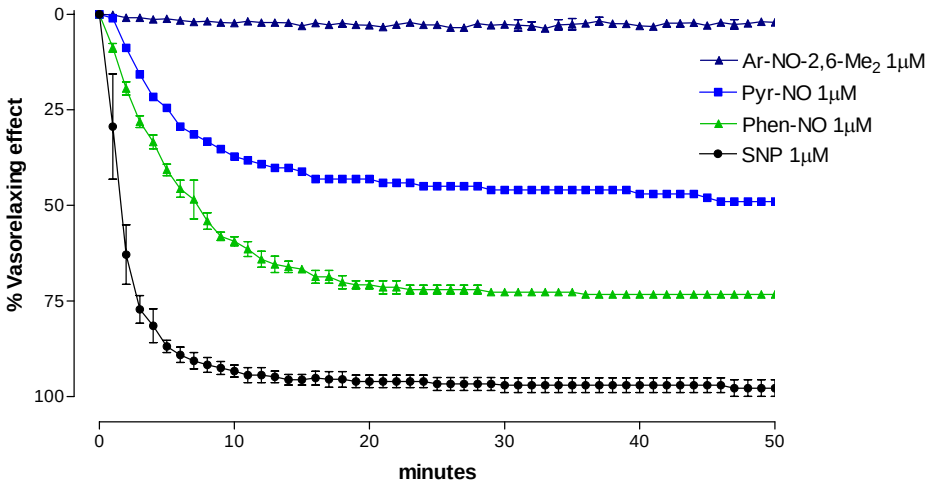

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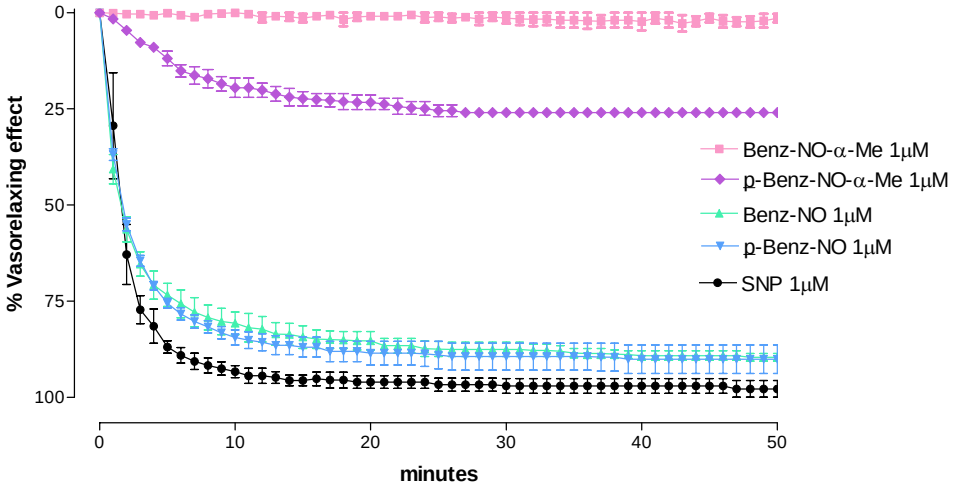
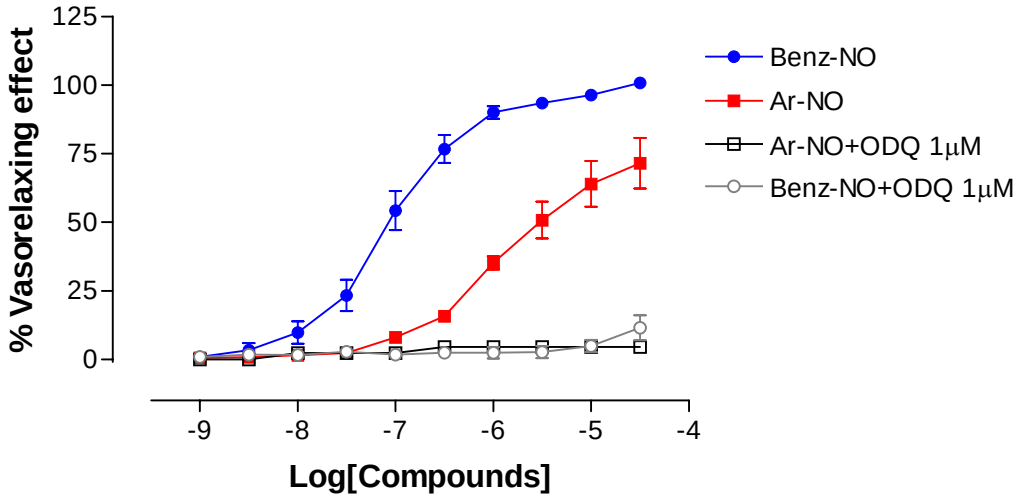


Figure 16. Representative examples of the influence of the guanylate cyclase inhibitor ODQ 1μM on the vasorelaxing effects of the tested compounds: concentration-response curves for compounds **1a** and **6a**, in the presence or in the absence of ODQ.



Discussion

Although this preliminary study has been focused on a limited number of lead compounds, some considerations on the structure-activity relationships could be hypothesised, thus concerning the influence of slight structural variations on the NO-releasing properties of the nitrooxy group.

As regards the carboxylic derivatives, a comparison of **Ar-NO** vs **p-Ar-NO** as well as **Ar-NO-α-Me** vs **p-Ar-NO-α-Me** showed that the *para* position ensured a more effective NO-release. In particular, **p-Ar-NO** exhibited levels of potency higher than those of **Ar-NO**; also the T25 value of **p-Ar-NO** was significantly lower than that of **Ar-NO**, indicating a more rapid release of NO. Although the couple of analogues **Ar-NO-α-Me** and **p-Ar-NO-α-Me** showed almost equivalent levels of potency and efficacy, a more rapid release of NO from **p-Ar-NO-α-Me** was highlighted by its T25 parameter, which was significantly lower than that

displayed by **Ar-NO-α-Me**. As regards the role played by the insertion of a methyl group into the nitrooxymethyl chain, this did not seem to exert any significant influence for the couple of meta-substituted analogues **Ar-NO** and **Ar-NO-α-Me**, while it seemed to have a negative impact for the para-substituted couple **p-Ar-NO** and **p-Ar-NO-α-Me**: compound **p-Ar-NO-α-Me** showed a lower level of potency than **p-Ar-NO,** and, consistently, its NO-release was slower. The insertion of two methyl groups into the aromatic ring (**Ar-NO-2,6-Me_2**) led to a decrease of potency (see **Ar-NO-2,6-Me_2** vs **Ar-NO**). Consistently, compound **Ar-NO-2,6-Me_2** (1μM) did not possess any significant vasorelaxing effect in the time-course protocol. The replacement of the benzene ring of **Ar-NO** with a pyridine system (**Pyr-NO**), as well as the replacement of the carboxy function of **Ar-NO** with a hydroxy group (**Phen-NO**), caused an improvement of both the vasorelaxing potency and the rate of NO-release. The benzylic derivatives **Benz-NO** and **p-Benz-NO** proved to possess the highest level of potency among the compounds tested, and their NO-release rates similar to that of SNP. In the benzylic series, the *para*-substituted compound **p-Benz-NO-α-Me** proved to be more potent NO-donor than the *meta*-substituted one **Benz-NO-α-Me**. However, the positive effect due to this structural feature was not evident for the couple of analogues **Benz-NO** and **p-Benz-NO**, which exhibited almost equivalent levels of pIC50 and T25. The negative influence due to the insertion of the methyl group into the nitrooxymethyl chain, already observed in the benzoic series, was even more evident in the benzylic ones (see **Benz-NO-α-Me** vs **Benz-NO** and **p-Benz-NO-α-Me** vs **p-Benz-NO**). In particular, compound **Benz-NO-α-Me** was found to be the less potent among all the compounds tested, and did not

exhibit any vasorelaxing effect in the time-course protocol, while compound **p-Benz-NO-α-Me** exhibited a poor vasorelaxing effect which did not allow a correct evaluation of T25.

Conclusion

This study aimed to develop some representative NO-donor structures, potentially useful as linkers to be added to native drugs, in order to obtain pharmacodynamic hybrids. In such hybrids, the presence of the two mechanisms of action was clearly the essential condition; however, a correct balancing of the two pharmacodynamic properties was another fundamental aspect. In particular, depending on the characteristics of the native drug (mechanism of action, posology, therapeutic indications), the release of NO should be correctly modulated in order to obtain well-calibrated levels of additive biological effects of NO itself. The availability of different NO-donor linkers possessing different NO-releasing rates, thus represented a necessary tool for this purpose.

The main findings of this work indicate that the shift of the nitrooxymethyl chain from the *meta*- to the *para*-position, both in benzoic and in benzylic derivatives may significantly influence the NO-donor properties, determining a more effective release. Furthermore, the insertion of a methyl group into the nitrooxymethyl chain, as well as the presence of two methyl groups on the aromatic system, determined a reduction in the rate of NO-release. On the other hand, it is reasonable to hypothesise that in a pharmacodynamic hybrid, the NO-releasing rate of a given NO-donor linker may be influenced by the presence of a further molecular portion (i.e. the native drug) linkers alone.

CHAPTER 3

*NO-SARTANS: A NEW CLASS OF PHARMACODYNAMIC HYBRIDS AS CARDIOVASCULAR DRUGS**

*The contents of chapter 3 have been already published:

Breschi M.C., Calderone V., Digiacomo M., Martelli A., Martinotti E., Minutolo F., Rapposelli S., Balsamo A. (2004) ***J. Med. Chem., 47; pp. 5597-5600.***

Breschi M.C., Calderone V., Digiacomo M., Macchia M., Martelli A., Martinotti E., Minutolo F., Rapposelli S., Rossello A., Testai L., Balsamo A. (2006) ***J. Med. Chem., 49; pp. 2628-2639.***

Introduction

The pharmacotherapy of hypertension involves several different classes of drugs; among these, the ones that have the renin-angiotensin system (RAS) as the target of their mechanism of action deserve special mention. ACE-inhibitors belong to this group, seeing that they act by inhibiting the Angiotensin Converting Enzyme (ACE), which is mainly responsible for the conversion of angiotensin I into angiotensin II. The latter is one of the most hypertensive substances in our organism: stimulation of the AT1-receptor by angiotensin II determines the release of aldosterone, which, by means of a reabsorption of Na^+ ions (and consequently of liquids) and a loss of K^+ ions, provokes hypertension. Furthermore, angiotensin II induces a direct vasoconstrictor action, which plays a significant role in hypertensive effects.

ACE is also involved in the mechanism of degradation of many other peptides, such as bradykinin. This peptide induces the release of endothelial nitric oxide (NO), a factor which plays a key role in the endogenous process of vasodilatation. The fact that ACE-inhibitors save bradykinin also leads to a vasorelaxing effect, which contributes to the anti-hypertensive action that is mainly due to the lack of angiotensin II production (Mombouli and Vanhoutte, 1992; Linz et al., 1995). Recent studies seem to indicate the possible existence of bradykinin-potentiating effects of ACE-inhibitors, which are independent of the reduction of bradykinin hydrolysis; this mechanism was initially demonstrated by using bradykinin analogues which were assumed to be ACE-resistant (Auch-Schwelk et al., 1993; Danser et al., 2000; Minshall et al., 1997; Minshall et al., 2000; Mombouli et al., 2002). This hypothesis is based on a “cross-talk” between ACE and bradykinin

type 2 (B_2) receptors (Marcic et al., 2000) (for example, forming a heterodimer), which leads to an up-regulation of the B_2 receptor (Minshall et al., 1997), probably accompanied by an up-regulation (Marin-Castano et al., 2002) and direct activation of bradykinin type 1 (B_1) receptors by ACE-inhibitors (Ignjatovic et al., 2002).

In view of the above-mentioned reasons, ACE-inhibitors represent a first-choice class in hypertension therapy. Furthermore, they also reveal an implication at the cardiac level: angiotensin II inhibits muscular apoptosis, thus provoking ventricular hyperplasia, which might aggravate a pre-existent cardiac failure.

However, ACE-inhibitors also frequently present an adverse side-effect which is invalidating in social life, and which drastically reduces the patient's compliance: this is coughing, which is the result of the ability of ACE-inhibitors to preserve bradykinin, a kinin that stimulates the cough-centre, from hydrolysis (Israili and Hall, 1992).

In the last decade, research on drugs which can replace ACE-inhibitors in hypertension therapy, without their collateral effects, has led to the discovery of sartans (Wexler et al., 1996). Drugs of this class are antagonists of angiotensin II at the AT1-receptor and block the action of angiotensin II in a potentially more complete way than ACE-inhibitors; as is known, there are other enzymes, besides ACE, which are able to contribute to angiotensin II production (Okunishi et al., 1993). Many comparative clinical trials have shown that sartans do not induce coughing because they do not prevent ACE from degrading bradykinin; in the past, they were employed when coughing was intolerable in a patient receiving therapy with ACE-inhibitors, but nowadays, in view of the evidence presented,

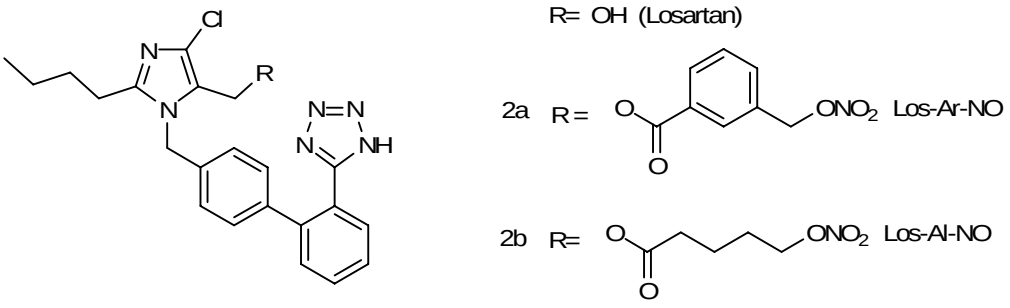
sartans can be viewed as a first-choice class of anti-hypertensive drugs (Tanser et al., 2000; Zorba Paster et al., 1998). However, sartans lack the enhancement of the NO-mediated vasorelaxing effect due to bradykinin preservation.

The importance of NO lies not only in its vasorelaxing action, but also in its potent inhibition of platelet and neutrophil aggregation in the endothelium (Moncada and Higgs, 1993; Loscalzo and Welch, 1995).

The above-mentioned biopharmacological considerations, together with the appreciable results obtained in different classes of NO-releasing drugs, prompted us to develop a new class of drugs, by adding a NO-donor group to a sartan molecule. This kind of chemical manipulation was likely to give the "native" sartan an additional NO-mediated, but bradykinin-independent, vasorelaxing effect, thus generating the original class of NO-sartans, pharmacodynamic hybrids possessing the properties of a typical AT1-antagonist and of a "slow NO-donor". The new compounds were expected to possess a pharmacodynamic profile very similar to that of ACE-inhibitors, but without their bradykinin-mediated adverse effects (Breschi et al., 2004).

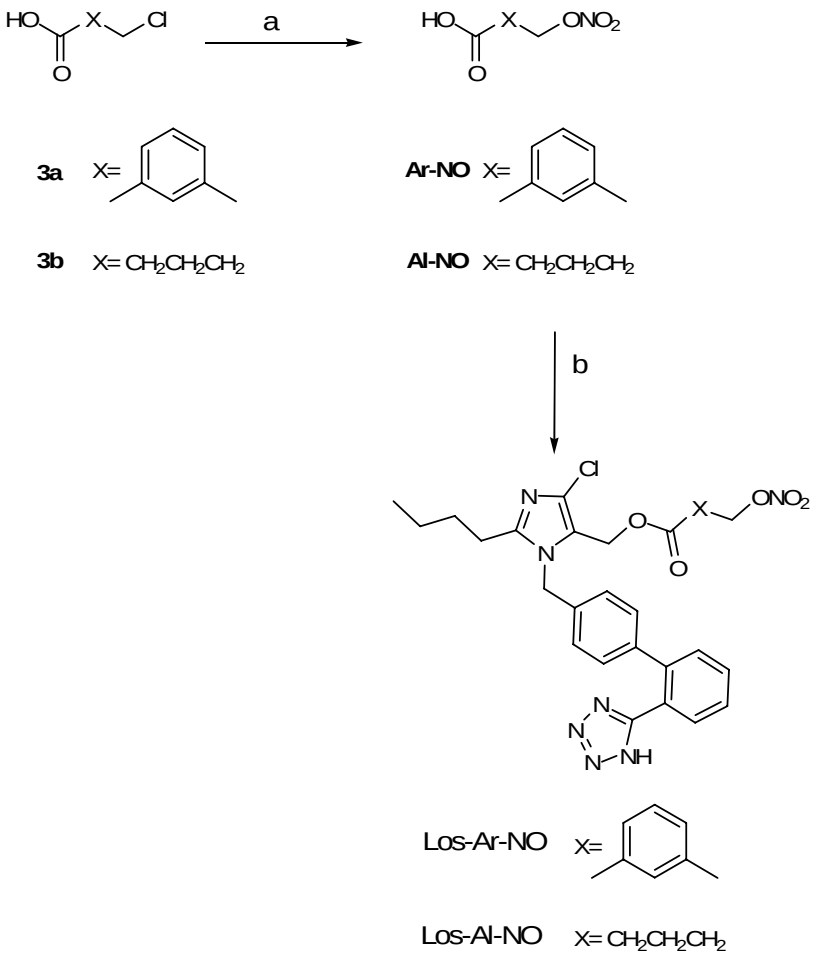
An examination of the structures of commercially available sartans showed that losartan possesses both the high activity and the molecular features, i.e. the presence of an easily esterifiable alcohol group, suitable to provide a template for our purpose. As regards the NO-donor moiety, the previously reported (Earl et al., 2004) *m*-nitrooxymethylbenzoate (**2a**, Figure 17) and the 5-nitrooxypentanoate (**2b**, Figure 17) were selected, based on the hypothesis of a different stability towards ester bond cleavage, depending on the aliphatic or aromatic nature of the compound.

Figure 17. Structure of losartan and general structure of the NO-releasing derivatives of losartan



The chemical pathway giving access to the final products **Los-Ar-NO** and **Los-Al-NO** is reported in Scheme 3.

Scheme 3.



Key to Scheme 3. [a]Key: (a) $AgNO_3$, CH_3CN, 1h, rt; (b) Losartan, DCC, THF, DMAP, 2h, rt.

m-(chloromethyl)benzoic acid (**3a**) or 5-chloropentanoic acid (**3b**) were converted into their nitrooxy-derivatives **Ar-NO** or **Al-NO** by treatment with silver nitrate in acetonitrile at room temperature and in the dark. Condensation of the nitro esters (**Ar-NO** and **Al-NO**) with losartan in tetrahydrofuran, in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and a catalytic amount of *N,N*-dimethylaminopyridine (DMAP), afforded the corresponding targets compounds **Los-Ar-NO** and **Los-Al-NO**.

Pharmacological studies were carried out on the two "pharmacodynamic hybrids" **Los-Ar-NO** and **Los-Al-NO**, by means of functional tests on vascular tissues, usually employed to determine NO-mediated responses (Villarroya et al., 1999) and AT1-antagonist activities (Morsing et al., 1999).

Pharmacological procedures (1)

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

In vitro protocols

The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250-350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding.

The aortae were immediately excised, freed of extraneous tissues and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41;

$NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with an unirecord microdynamometer (Buxco Electronics).

Evaluation of the NO-releasing properties

After an equilibration period of 60 minutes, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted vascular rings. A relaxation < 10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation ≥ 10% (i.e. significant presence of the endothelium), were discarded. In the first series of experiments, we investigated the possible NO-releasing effect of the tested compounds.

From 30 to 40 minutes after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM) and when the contraction reached a stable *plateau*, 3-fold increasing concentrations of **Los-Ar-NO**, **Los-Al-NO** and **Ar-NO** (1nM-10μM) were added.

When required by the experimental design, the administration of the tested compounds was preceded by the pre-incubation (20 min) of eserin 3μM.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 minutes.

The same experiments were carried out in the presence of a well-known guanylate-cyclase inhibitor: ODQ 1μM which was incubated in aortic preparations after the endothelium removal confirmation.

Evaluation of the AT1-antagonism

In the second series of experiments the possible AT1 antagonist activity of the selected substances was examined. After the endothelium removal confirmation, the tested compound **Los-Ar-NO** and the reference AT1 antagonist losartan were incubated at the concentration of 0.1 μM.

When required by the experimental protocol, the administration of the tested compounds was preceded by the pre-incubation (20 min) of eserin 3μM.

After an incubation period of 30 minutes, aortic preparations were treated with angiotensin II, using 3-fold increasing concentrations (0.1 nM-1 μM).

Both **Los-Ar-NO** 0,1μM and **Los-Al-NO** 0,1μM did not evoke significant vasorelaxing effects. Nevertheless, in order to exclude a possible involvement of a NO-mediated functional antagonism against the contractile effects of angiotensin II, in previous experiments the study on the AT1-antagonism were carried out in the presence of ODQ 1μM.

These experiments furnished results analogous to those obtained in the absence of ODQ.

Materials.

Substances used in the experimental protocols were KCl (Carlo Erba) dissolved (3M) in Tyrode solution, acetylcholine chloride (Sigma) dissolved (0.1 M) in EtOH 95% and further diluted in bidistillated water. Angiotensin II (Sigma) was dissolved (100 μM) and diluted in bi-distilled water. ODQ (Sigma) was dissolved (1 mM) in EtOH 95% and further diluted in Tyrode solution. Losartan was dissolved (10 mM) in dimetilsulfoxide (DMSO), whereas the following dilutions

were dissolved in Tyrode solution. Compounds **Los-Ar-NO, Los-Al-NO** and **Ar-NO** were dissolved (10 mM) in DMSO and further diluted in Tyrode solution.

All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicles.

Data analysis.

The vasorelaxing efficacy was evaluated as maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. When the limit concentration 10 μM (the highest concentration, which could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean ± standard error, for 5-10 experiments. Student *t* test was selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

As regards the AT_1-antagonism, the angiotensin II-contracting effects, expressed as efficacy and potency, were evaluated as percentage (%) of the previous KCl 30mM-induced contraction.

The parameter of efficacy, corresponding to the maximal contractile effect, was calculated as E_{max} (mean ± standard error, from 6-12 experiments).

The parameter of potency of angiotensin II was calculated as EC_{50}, corresponding to the molar concentration of angiotensin II necessary to evoke $1/2E_{max}$.

The antagonist potency was calculated by Gaddum equation:

$$Kb = B/(DR-1)$$

In which:

Kb represents the antagonist/receptor dissociation constant and B is the antagonist concentration.

While,

$$DR = EC_{50}(\text{in the presence of B})/EC_{50}(\text{in control conditions})$$

In vivo protocols.

The effects of the compounds were also tested on male 10 weeks-old SHRs (spontaneous hypertensive rats) (250 g).

In this protocol the compounds were administered orally (in drinking water) or sub-cutaneously to two set of four groups each composed by three rats.

Oral administration

We estimate (on the basis of previous observations) that the daily water intake for a single rat was about 50 mL.

1) The first group (control-group) received the vehicle (DMSO 1%, in drinking water).

2) The captopril-group received captopril 50 mg/Kg/die dissolved in the vehicle.

3) The losartan-group received losartan 10 mg/Kg/die dissolved in the vehicle.

4) The **Los-Ar-NO**-group received **Los-Ar-NO**-compound 14.2 mg/Kg/die (equimolar to losartan 10 mg/Kg/die).

Sub-cutaneous administration

1) The first group (control-group) received the vehicle (DMSO 1%, in dorsal sub-cutaneous injection, about 0,25-0,30 ml).

2) The captopril-group received captopril 25 mg/Kg/die dissolved in the vehicle.

3) The losartan-group received losartan 5 mg/Kg/die dissolved in the vehicle.

4) The **Los-Ar-NO**-group received **Los-Ar-NO** 7.1 mg/Kg/die (equimolar to losartan 5 mg/Kg/die).

Common procedures

The experimental protocol was divided in a previous period of two weeks, during which the rats were daily conditioned to entry and to remain in a containment box. After the conditioning time, the animal tails were exposed to a 40 minutes of irradiation with an I.R. lamp to determine a vasodilatation of tail vessels. Systolic blood pressure values were recorded with the "tail-cuff" method by a BP recorder (Ugo Basile 58500).

All the four groups, were subjected to a four weeks treatment and to three measurements (on alternate days, at 9.00 a.m.) in each week.

Finally we also examined a group of three male normotensive Wistar rats (250g) receiving only drinking water; in these animal, after one week of conditioning, systolic blood pressure was recorded 3 times (on alternate days, at 9.00 a.m.) within only one week.

Results and Discussion

These two compounds induced full vasorelaxing effects (Efficacy = 92 ± 6% and 95 ± 8%, for **Los-Ar-NO** and **Los-Al-NO**, respectively; pIC_{50} = 5.55 ± 0.09 and 5.89 ± 0.14, for **Los-Ar-NO** and **Los-Al-NO**, respectively) which were strongly inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) 1μM, an inhibitor of guanylate cyclase, as to be expected in the case of an NO release effect (Efficacy = 34±3% and 23±8%, for **Los-Ar-NO** and **Los-Al-NO**, respectively; pIC_{50} values not calculable).

Also the AT1-antagonist properties of nitrooxy-derivatives **Los-Ar-NO** and **Los-Al-NO** were investigated and compared with those of the reference AT1-antagonist losartan.

The concentration-contractile response curve for angiotensin II was shifted parallel to the right by **Los-Ar-NO** and **Los-Al-NO**, with Kb values of 6 nM and 16 nM, respectively. Under the same experimental conditions, losartan displayed a Kb value of 4 nM, substantially analogous to the value recorded for **Los-Ar-NO** and slightly lower than that of **Los-Al-NO**.

Compound **Los-Ar-NO** was selected in order to obtain further information, in an attempt to understand the pharmacokinetic steps leading to the pharmacodynamic features observed.

In order to evaluate whether the vasorelaxing properties of **Los-Ar-NO** are due to the release of NO from the whole molecule or from the side chain (**Ar-NO**), after its hydrolytic removal from losartan, the vasorelaxing effects of **Ar-NO** were evaluated by functional tests.

Compound **Ar-NO** showed vasorelaxing effects lower than **Los-Ar-NO**, both in efficacy and in potency (Efficacy = 67 ± 12%; pIC_{50} = 4.66 ± 0.13).

This first finding, which seems to indicate that the NO-release from **Los-Ar-NO** is more rapid, and thus that it precedes the possible hydrolysis of the ester link between losartan and **Ar-NO**, is confirmed by the results obtained in the presence of eserin (3μM, an inhibitor of esterases) (Gilmer et al., 2002).

As expected, the presence of the esterase-inhibitor did not alter the vasorelaxing effects of **Ar-NO** (Efficacy = 71 ± 6%; pIC_{50} = 4.64 ± 0.07); significantly, the effects of **Los-Ar-NO** were not influenced by eserin, either (Efficacy = 91 ± 3; pIC_{50} = 5.37 ± 0.05), confirming that the release of NO from this compound does not require previous hydrolytic removal of the side chain.

An analogous experimental strategy was employed to determine whether the AT1-antagonist properties of **Los-Ar-NO** are due to the whole molecules or to losartan (after the hydrolytic removal of the side chains).

As reported above, the AT1-antagonist potencies of losartan and of **Los-Ar-NO** were quite indistinguishable, and in our opinion, this experimental evidence already tended to suggest that the antagonism was exerted by losartan itself, after the hydrolytic removal of the side chain, as desired for our purposes.

The experimental confirmation of this hypothesis was provided by the use of eserin: in the presence of this esterase-inhibitor, the antagonist potency of **Los-Ar-NO** was dramatically lowered (Kb = 40 nM), while eserin did not influence the AT1-antagonist potency of losartan (Kb = 5 nM).

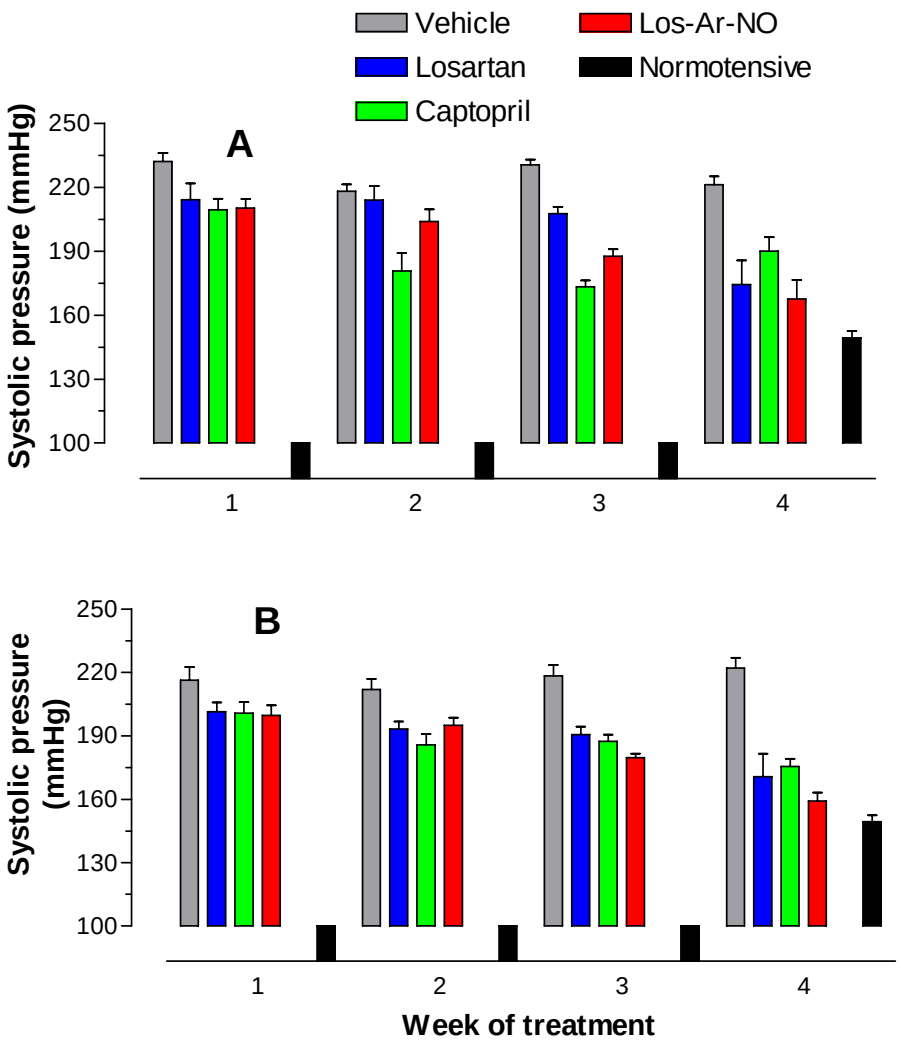
These initial functional data suggest that the main aim of this work, i.e. the creation of the original pharmacological class of NO-sartan AT1-antagonists/NO-donor hybrids, has been satisfactorily achieved.

Furthermore, an "exploratory" in vivo protocol was carried out on **Los-Ar-NO**, in order to obtain a preliminary indication about the possible profile of the anti-hypertensive action of a compound of this new pharmacological class, in comparison with that of an AT1-antagonist and an ACE-inhibitor.

Consequently, compound **Los-Ar-NO**, losartan and captopril were administered orally or sub-cutaneously to spontaneously hypertensive rats (SHR) for four weeks, recording the systolic blood pressure, by the "tail cuff" method (Krege et al., 1995).

The order of magnitude of the oral doses of captopril and losartan was selected on the basis of similar experimental protocols, described in literature (Rodrigo et al., 1997), while **Los-Ar-NO** was administered at a dose equimolar to that of losartan. For sub-cutaneous administration, the doses were reduced by one half. In both these sets of experimental conditions, all compounds had practically equivalent effects, causing a significant reduction in the systolic pressure, which was lowered almost to the levels of reference normotensive animals (Figure 18).

Figure 18. Histograms indicate the systolic blood pressure values recorded in the first four weeks of oral (**A**) or sub-cutaneous (**B**) pharmacological treatment with the vehicle, losartan, captopril and **Los-Ar-NO**. Systolic pressure values of reference normotensive animals are also shown. Vertical bars indicate the standard error.



In our opinion, this result was of fundamental importance, since the ineffectiveness or of a low effectiveness of **Los-Ar-NO** was considered to be a critical factor, capable of invalidating the rational basis of our work.

In conclusion, the results of *in vitro* and *in vivo* studies confirm that these NO-sartans are actually pharmacodynamic hybrids possessing both the AT1-

antagonist activity of sartans and the ancillary NO-releasing property of an NO-donor.

In addition, they seem to possess anti-hypertensive properties not inferior to those of sartans, and it is reasonable to conclude that this new class of drugs strengthens the action of the "native" sartans, integrating the satisfactory anti-hypertensive effects of the "native" drugs with all the other beneficial roles played by NO in the cardiovascular system (Breschi et al., 2004).

On the basis of the consideration that losartan and its active metabolite (**EXP-3174**) (Figure 19) possess both the high activity and the molecular features (i.e. the presence of an easily esterifiable group) useful for our purposes, a series of new dual molecule, in which losartan itself is linked to different molecular portions bearing a nitric ester moiety, were synthesised (**Los-Al-NO, Los-Ar-NO, Los-p-Ar-NO, Los-Ar-NO-α-Me, Los-p-Ar-NO-α-Me, Los-Ar-NO-2,6-Me$_2$, Los-Pyr-NO, Los-NO**) (Figure 20).

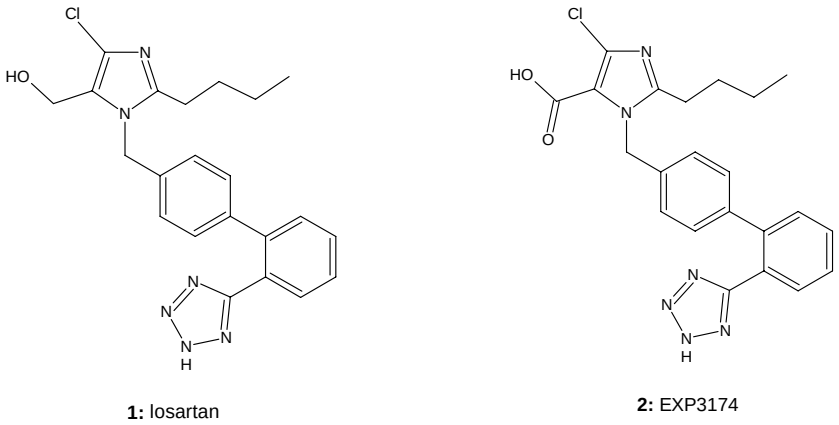


Figure 19. Losartan and its active metabolite: EXP 3174

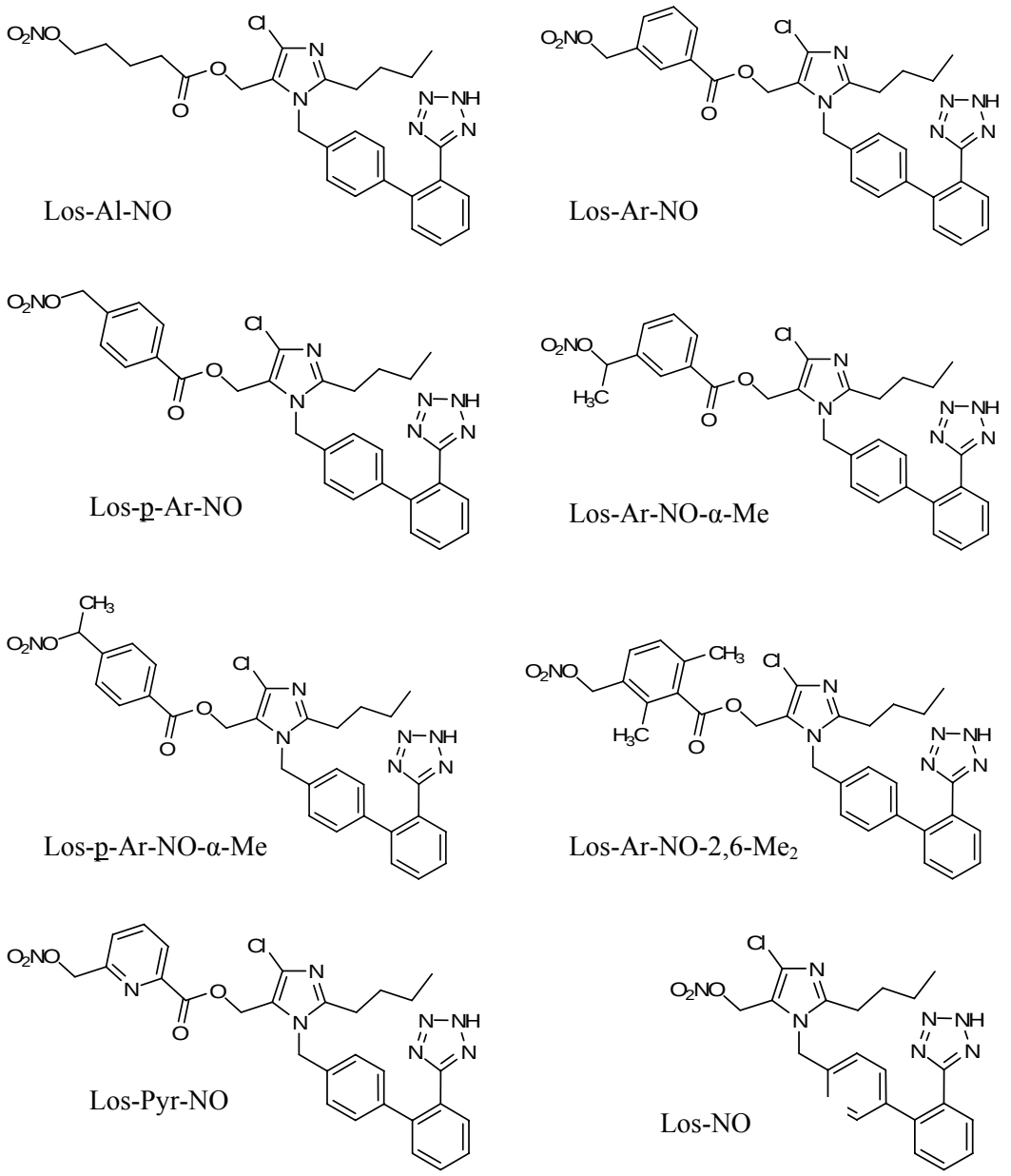


Figure 20. The series of new duel molecule in which losartan is linked to different molecular portions bearing a nitric ester moiety.

Furthermore, the hybrids in which an aromatic nitric ester is linked to the active metabolite of losartan and one in which the nitric ester function is directly linked

to losartan itself were synthesised (**Exp-Phen-NO, Exp-Benz-NO, Exp-p-Benz-NO**) (Figure 21).

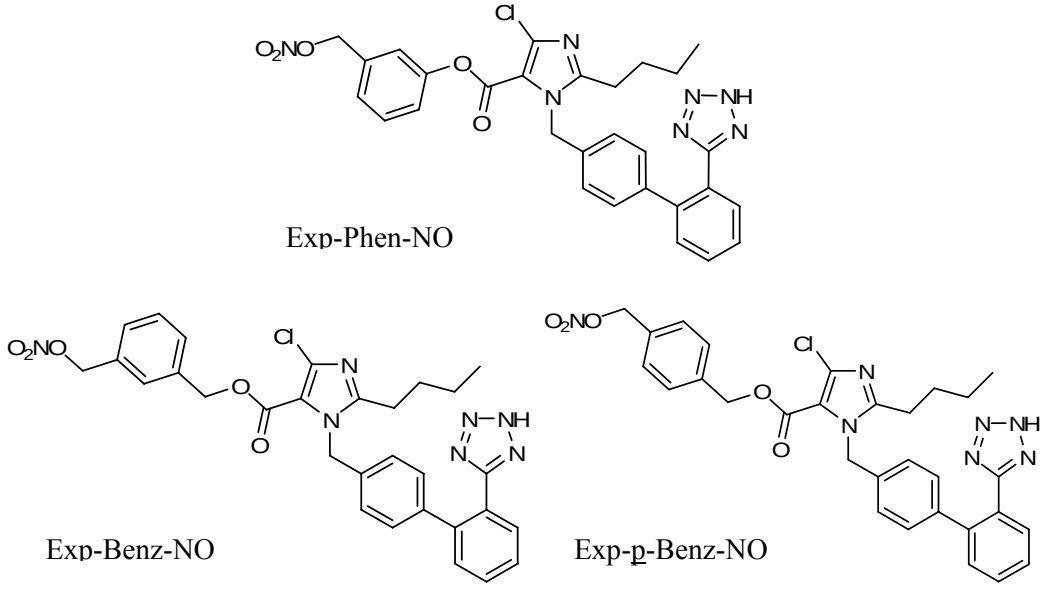


Figure 21. The series of new duel molecule in which EXP 3174 is linked to different molecular portions bearing a nitric ester moiety.

The "NO-donor linkers" used in this work, present a pyridine system or methyl group(s) directly linked to the aromatic systems or to the carbon adjacent to the nitric ester function. These linkers were selected on the basis of the hypothesis that steric and/or electronic differences, due to their different molecular structures, may modulate the rate of NO release and therefore affect the biopharmacological responses.

Pharmacological procedures (2)

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

In vitro vascular protocols

The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250-350 g).

After light ether anaesthesia, the rats were sacrificed by cervical dislocation and bleeding.

The aortas were immediately excised and freed from extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five-mm-wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37°C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with an unirecord microdynamometer (Buxco Electronics).

After an equilibration period of 60 min, endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted rings. A relaxation < 10% of the KCl-induced contraction was considered to be indicative of an acceptable lack of the endothelial layer, while the organs showing a relaxation ≥ 10% (i.e. significant presence of the endothelium) were discarded.

NO-mediated vasorelaxing effect

From 30 to 40 min after the confirmation of endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30mM), and when

the contraction reached a stable plateau, 3-fold increasing concentrations of the test substances (1nM-100μM) were added.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 min.

The same experiments were carried out in the presence of a well-known guanylate-cyclase inhibitor; ODQ 1μM, which was incubated in aortic preparations after confirmation of endothelium removal.

AT_1 antagonist activity

From 30 to 40 min after confirmation of endothelium removal, the test compounds were incubated at a concentration of 0.1μM. As the concentration 0.1μM of compounds **Los-Ar-NO** and **Los-p-Ar-NO** already induced a significant vasorelaxing effect, **Los-Ar-NO** and **Los-p-Ar-NO** were incubated together with ODQ 1μM, in order to avoid any influence of the NO-mediated effects on the contractile response evoked by AII.

Then, after an incubation period of 20 or 60 min, aortic preparations were treated with AII, using 3-fold increasing concentrations from 0.1 nM-1μM.

In parallel sets of experiments, the control concentration-contractile response curves for AII were obtained after the pre-incubation of only the vehicle (or vehicle and ODQ 1μM for **Los-Ar-NO** and **Los-p-Ar-NO**).

Time-course of NO-mediated vasorelaxing effect

From 30 to 40 min after confirmation of endothelium removal, aortic preparations were contracted with a single concentration of KCl 30mM, and after a stable

plateau had been reached, a single concentration (1μM) of the compounds was added. The vasorelaxing effect of the added compounds was monitored for 50 min.

Data analysis

The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30mM. When the limit concentration 100 μM (the highest concentration that could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30mM, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as the negative logarithm of the molar concentration of the test compounds, evoking a 50% reduction of the contractile tone induced by KCl 30mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means ± standard error, for 5-10 experiments. Student *t* test was selected for statistical analysis, and $P < 0.05$ was considered to be indicative of a significant statistical difference. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

As regards the AT_1-antagonism, the AII-contracting effects, expressed as efficacy and potency, were evaluated as a percentage (%) of the previous KCl 30mM-induced contraction.

The parameter of efficacy, corresponding to the maximal contractile effect, was calculated as E_{max} (mean ± standard error, from 6-12 experiments).

The parameter of potency of AII was calculated as EC50, corresponding to the molar concentration of AII necessary to evoke $1/2E_{max}$.

The antagonist potency was calculated by Gaddum's equation: Kb=B/(DR-1), in which Kb represents the antagonist/receptor dissociation constant and B is the antagonist concentration, while DR=EC50(in the presence of B)/EC50(in control conditions).

In vitro cardiac protocols

Adult male Wistar rats (260-350 g) were randomly assigned to one of three groups. In the first group (n=5), animals were treated with an intra-peritoneal (i.p.) injection (about 0.3 mL) of compound **Losartan** (15.0 mg/Kg), the second group (n=5) was treated with the equimolar dose of compound **Los-Ar-NO** (21.3 mg/Kg i.p.), and the third group (n=5) received the vehicle (DMSO).

After 2 h, all the animals were anaesthetised with sodium pentobarbital (100 mg/Kg i.p.) and heparinised (100 UI i.p.) to prevent blood clotting. After the opening of the chest, the hearts were quickly excised and placed in a 4°C Krebs solution (composition mM: $NaHCO_3$ 25.0, NaCl 118.1, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2.2H_2O$ 1.6, KH_2PO_4 1.2, glucose 11.5) equilibrated with 95% O_2 5% CO_2, to stop the contraction and to reduce oxygen consumption. Rapidly, the ascending aorta was cannulated and hearts mounted on a Langendorff apparatus, then the perfusion with Krebs solution (thermostated at 37°C and continuously bubbled

with a gas mixture of 95% O_2 and 5% CO_2) was started at constant pressure (70-80 mmHg). The above procedure was executed within 2 min.

A water-filled latex balloon connected to a pressure transducer (Bentley Trantec, mod 800) was introduced into the left ventricle via the mitral valve and the volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5-10 mmHg during initial equilibration. The heart rate (HR) and left ventricular developed pressure (LVDP) were monitored by a Biopac system (California, USA) and the parameter of Rate Pressure Product (RPP) was calculated as RPP = HR x LVDP.

The coronary flow (CF) was measured volumetrically and was expressed as mL of the perfusate collected in 1 min.

After a 30 min equilibration pre-ischaemic period, the hearts were subjected to 30 min of global ischaemia (no flow), then they were reperfused for 30 min (reperfusion period).

The functional parameters of RPP and CF were measured before the global ischaemia and at 5 min intervals during the reperfusion time. The values of post-ischaemic RPP and of post-ischaemic CF, recorded in the reperfusion time, were reported as a percentage of the respective pre-ischaemic values. Hearts showing severe arrhythmia were excluded from the experiments.

In vitro anti-platelet activity

Male Wistar rats (250-300g) were anaesthetised with sodium pentobarbital (100mg/Kg, i.p.), the chest was opened and the blood (7-10 mL) was obtained through intracardiac puncture.

Blood was collected into 5 mL plastic syringes containing 3.8% sodium citrate (1:9 v/v) and then centrifuged.

Human venous blood was obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers were informed that blood samples were obtained for research purposes and that their privacy would be protected.

Platelet-rich plasma (PRP) was obtained by centrifugation at 80g for 40 min at room temperature. Then the top layer, PRP, was removed while the residual blood sample was centrifuged at 1550g for 25 min in order to obtain platelet-poor plasma (PPP). The PRP platelet number was adjusted at about 6×10^5 platelets/μL, through dilution with normal saline (NaCl 0.9%).

Platelets aggregation was determined, in diluted PRP, by an optical method, using a turbidimetric aggregometer (Elvi 840, Milan). In the aggregometer, contents of cuvettes (500μL) were maintained at 37°C and stirred constantly at 1000 r.p.m. Changes in light transmission through the diluted PRP and PPP were used for calibration, and represent minimum (0%, no aggregation) and maximum (100%, full aggregation) light transmission, respectively.

In preliminary experiments concentration-response curves to the aggregating agent (ADP) were obtained, and the 5μM ADP concentration was selected. Aggregation % was recorded as increased light transmission after the addition of the aggregating stimulus and has been expressed as mean ± standard error, from groups of 10-12 samples (the samples of each group were collected from at least 3 different individuals). The inhibitory effects of compounds **Losartan** (100μM) and **Los-Ar-NO** (100μM) on the ADP 5μM-induced aggregation were evaluated. The tested compounds were added 2 min before the administration of ADP 5μM.

Control responses were obtained in the presence of drug vehicle only (DMSO). The final concentration of DMSO was 0.5%.

In vivo protocols

The effects of the compounds were also tested on male 10-week-old SHRs (spontaneously hypertensive rats), (250g).

In this protocol, the test substances were administered orally, in drinking water, or sub-cutaneously, to two sets of four groups, each composed of three rats. The order of magnitude of the oral doses of captopril and losartan was selected on the basis of similar experimental protocols, described in literature (Rodrigo et al., 1997). **Los-Ar-NO** was administered at a dose equimolar to that of losartan. For sub-cutaneous administration, the doses were reduced by one-half.

Oral administration

We estimated that the daily water intake for a single rat was about 50 mL.

1) The first group (control-group) received the vehicle (DMSO 1%, in drinking water).

2) The captopril-group received captopril 50mg/Kg/die dissolved in the vehicle.

3) The losartan-group received losartan 10mg/Kg/die dissolved in the vehicle.

4) The **Los-Ar-NO**-group received **Los-Ar-NO** 14.2 mg/Kg/die (equimolar to losartan 10mg/Kg/die).

Sub-cutaneous administration

1) The first group (control-group) received the vehicle (DMSO 1%, in a dorsal sub-cutaneous injection, about 0.25-0.30 mL).

2) The captopril-group received captopril 25 mg/Kg/die dissolved in the vehicle.

3) The losartan-group received losartan 5mg/Kg/die dissolved in the vehicle.

4) The **Los-Ar-NO**-group received **Los-Ar-NO** 7.1 mg/Kg/die (equimolar to losartan 5mg/Kg/die).

Common procedures

The experimental protocol was divided: after an initial period of two weeks, during which the rats were daily conditioned to enter and to remain in a containment box, the animals' tails were exposed to 40 minutes of irradiation with an IR lamp to determine a vasodilation of tail-vessels. Systolic blood pressure values were recorded with the "tail-cuff" method by a BP recorder (Ugo Basile 58500).

All the four groups were subjected to four weeks' treatment and to three measurements a week (on alternate days).

Finally, we also examined a group of three male normotensive Wistar rats (250g) which received only drinking water; in these animals, after one week of conditioning, systolic blood pressure was recorded 3 times in only one week.

At the end of all the *in vivo* procedures, we evaluated cardiac and ventricular hypertrophy in SHRs: we recorded the body weight of each animal and then, after light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding.

The whole hearts were immediately excised, freed from extraneous tissues and then subdivided into left and right ventricles and atria. After rapid but careful washing and drying, we recorded the weights of left ventricle, right ventricle and atria, and we calculated the following ratios, as described in literature (Lassila et al., 2003)

- Heart weight/body weight (g/Kg)

- Left ventricle weight/body weight (g/Kg)
- Right ventricle weight/body weight (g/Kg)

The same ratios were also calculated in the sample (n=3) of normotensive animals, not submitted to any pharmacological treatment.

Materials

The substances used in the pharmacological experimental protocols were KCl (Carlo Erba) dissolved (3M) in Tyrode solution, Acetylcholine chloride (Sigma) dissolved (0.1 M) in EtOH 95% and further diluted in twice-distilled water;

Angiotensin II (Sigma) was dissolved (1 mM) in twice-distilled water and then diluted;

ODQ (Sigma) was dissolved (1 mM) in EtOH 95% and further diluted in Tyrode solution;

losartan was dissolved (10 mM) in DMSO, whereas the following dilutions were dissolved in Tyrode solution.

The reference NO-releasing drug SNP (Sigma) was dissolved (10 mM) in DMSO and then diluted in Tyrode solution. Sodium pentobarbital (Sessa) was dissolved in twice-distilled water. Heparin Vister was purchased by Pfizer as injectable preparation. ADP (Sigma) was dissolved in twice-distilled water.

All the synthesised compounds were dissolved (10 mM) in DMSO and further diluted in Tyrode solution.

All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicles.

Pharmacological results

NO-mediated vasorelaxing activity

All the tested compounds (**Los-Al-NO, Los-Ar-NO, Los-p-Ar-NO, Los-p-Ar-NO-α-Me, Los-Ar-NO-2,6-Me_2, Los-Pyr-NO, Los-NO**), with the only exception of **Los-Ar-NO-α-Me** which exhibited only a partial efficacy, evoked concentration-dependent vasorelaxing responses, with full efficacy, on rat aortic rings pre-contracted by KCl 30 mM (Table 4).

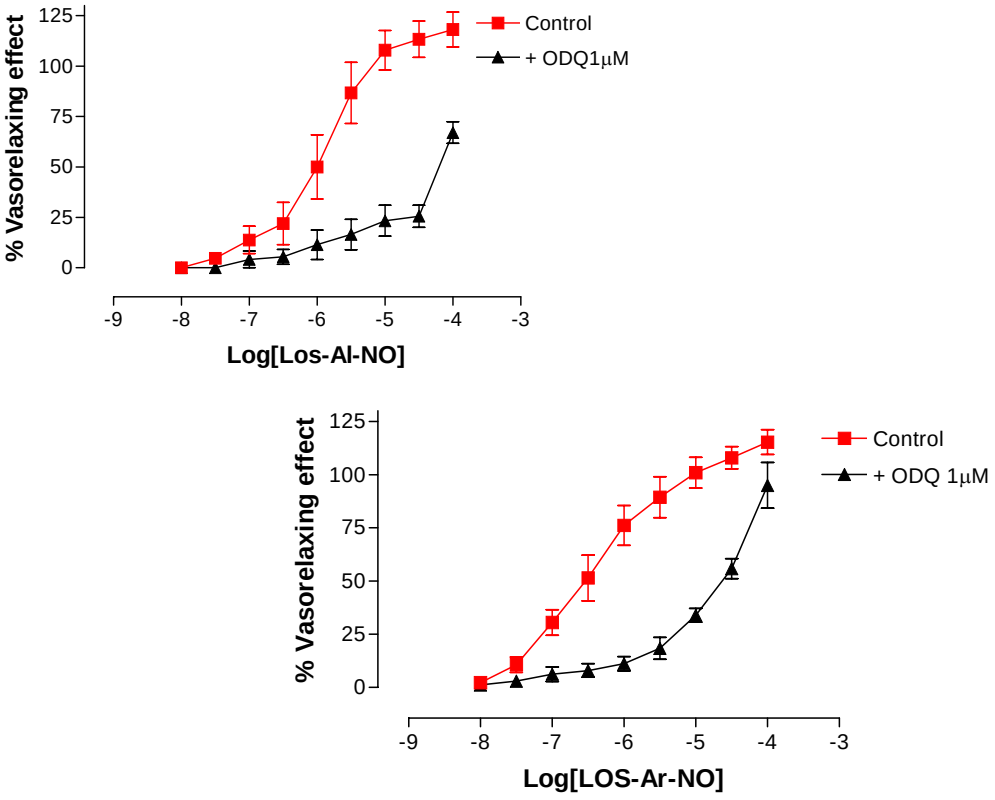
Compound	pIC_{50}	Emax %
Los-Al-NO	6.07 ± 0.11	100
Los-Ar-NO	6.56 ± 0.08	100
Los-p-Ar-NO	7.18 ± 0.03	100
Los-Ar-NO-α-Me	Not calculable	47 ± 1
Los-p-Ar-NO-α-Me	5.11 ± 0.03	100
Los-Ar-NO-2,6-Me_2	4.49 ± 0.02	100
Los-Pyr-NO	4.91 ± 0.06	100
Exp-Phen-NO	5.22 ± 0.08	100
Exp-Benz-NO	6.18 ± 0.03	100
Exp-p-Benz-NO	6.56 ± 0.02	100
Los-NO	4.82 ± 0.07	100

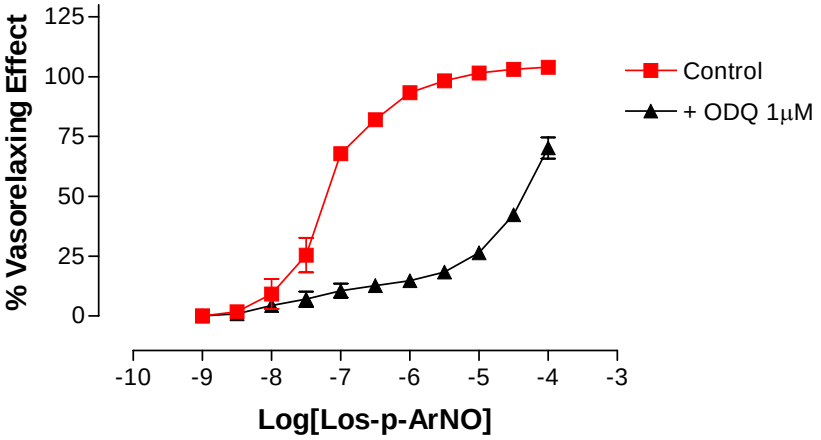
Table 4. The NO-mediated vasorelaxing efficacy of the synthesised compounds was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. The parameter of potency was expressed as pIC_{50}, calculated as the negative logarithm of the molar concentration of the test compounds, evoking 50% reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds

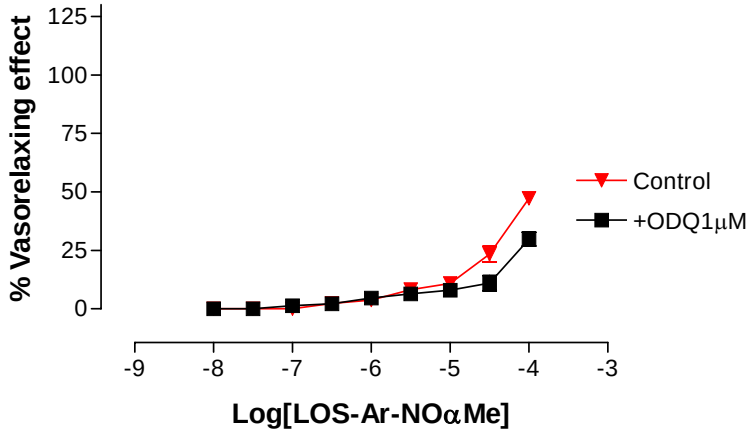
showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means ± standard error, for 5-10 experiments.

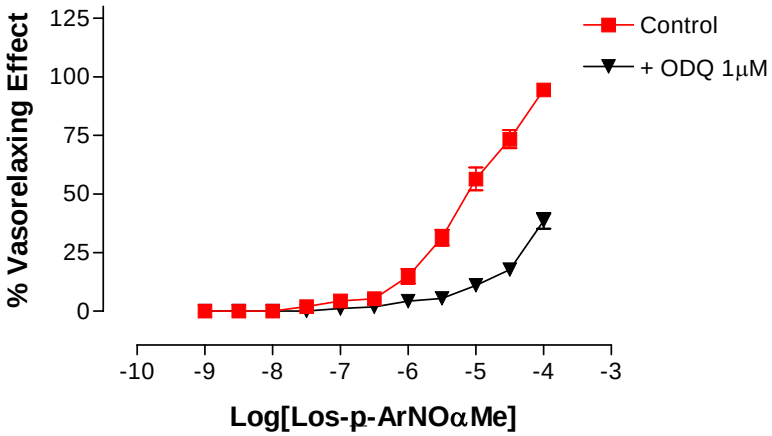
All these responses were significantly antagonised by the inhibition of guanylate cyclase, achieved by the administration of 1-*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxalin-1-one (ODQ) 1μM, thus indicating that the vasorelaxing effect was due to the release of NO from the hybrid drugs, and therefore to the triggering of the NO-cGMP pathway.

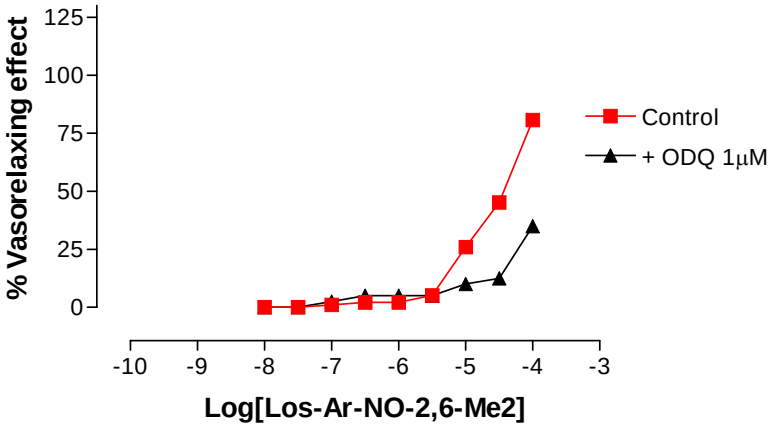
In particular, compounds **Exp-Benz-NO, Exp-p-Benz-NO** showed a vasorelaxing activity with potency parameters almost comparable with those exhibited by **Los-Al-NO** and **Los-Ar-NO**. Compound **Los-p-Ar-NO** showed the strongest vasorelaxing activity, with a potency parameter (pIC_{50}) higher than those exhibited by the previously described pioneer compounds **Los-Al-NO** and **Los-Ar-NO** (Figure 22A/B).

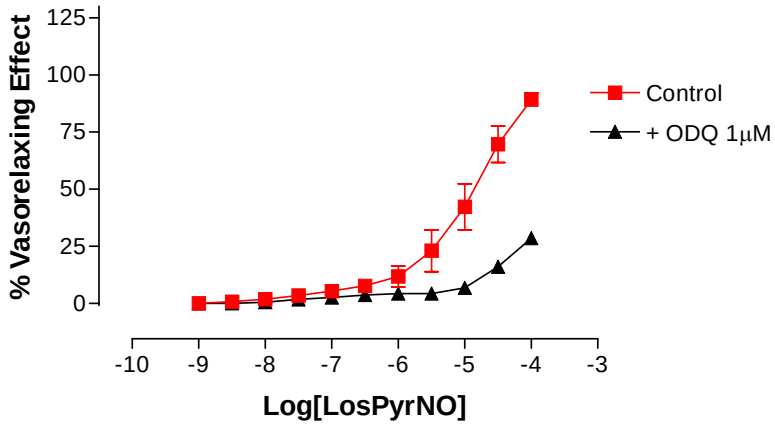



% Vasorelaxing Effect
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
-3
Log[Los-p-ArNO]
Control
+ ODQ 1μM


% Vasorelaxing effect
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
Log[LOS-Ar-NOαMe]
Control
+ODQ1μM


% Vasorelaxing Effect
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
-3
Log[Los-p-ArNOαMe]
Control
+ ODQ 1μM





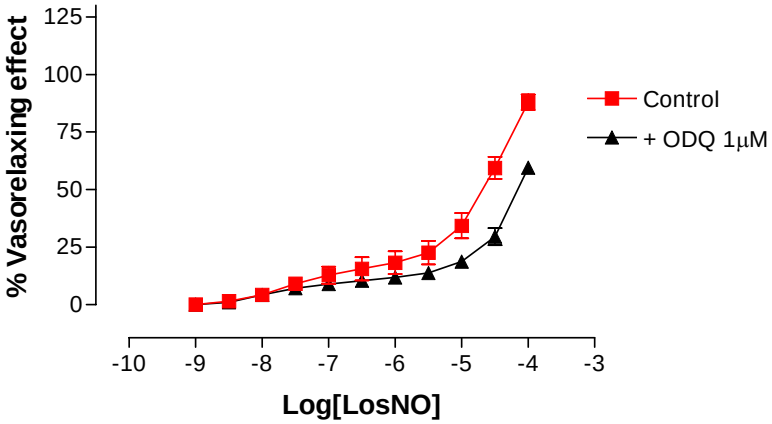


Figure 22A. Concentration-vasorelaxing effect curves of hybrid derivatives of Losartan. X Axis : Logarithm of the molar concentration of the test compounds; Y Axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM; Bars: standard errors.

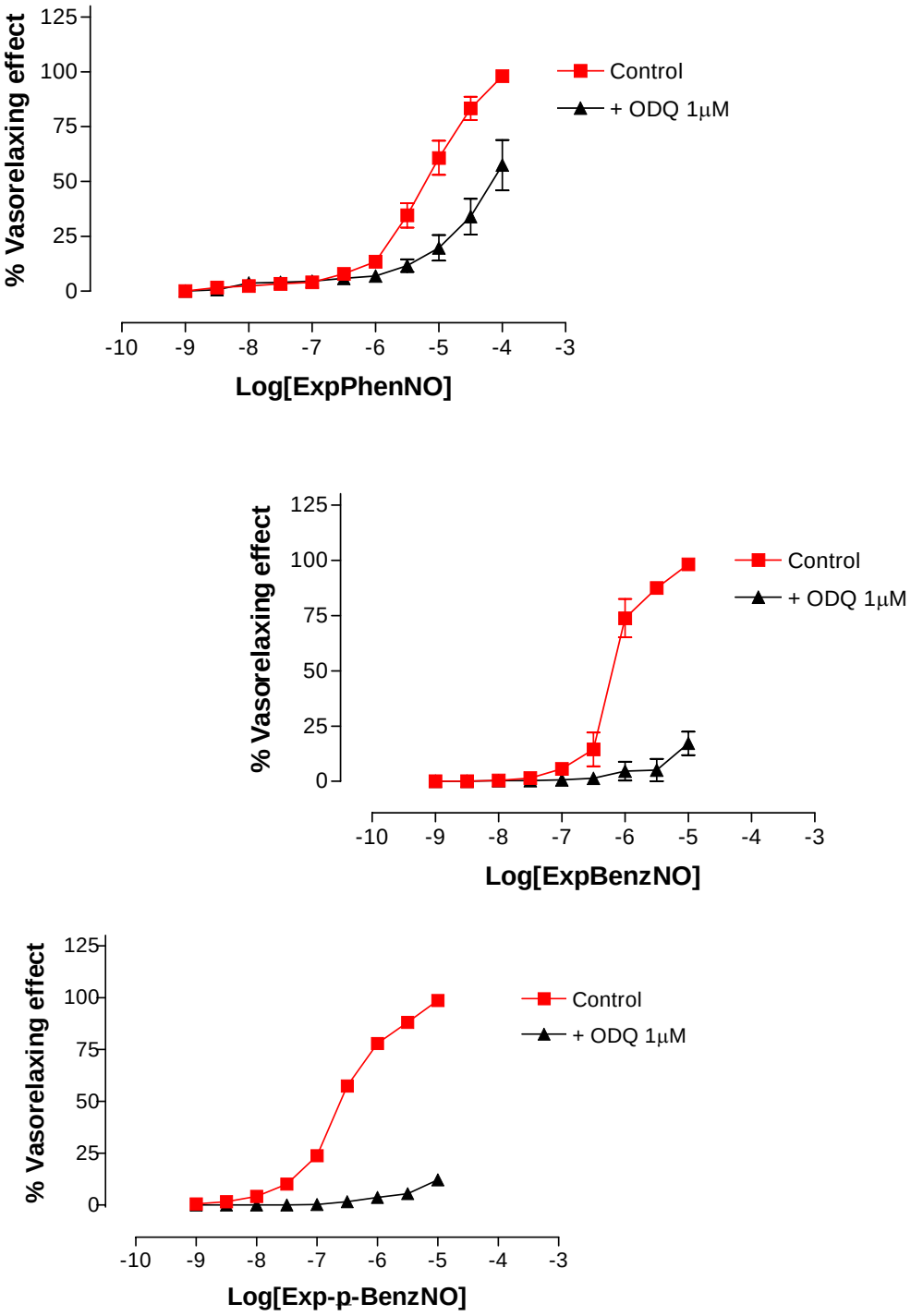


Figure 22B. Concentration-vasorelaxing effect curves of hybrid derivatives of EXP 3174. X Axis : Logarithm of the molar concentration of the test compounds; Y Axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM; Bars: standard errors.

In comparison with **Los-Al-NO** and **Los-Ar-NO**, compounds **Los-p-Ar-NO-α-Me** and **Exp-Phen-NO** showed a moderate decrease in the pIC_{50} values (about one order of magnitude), while **Los-Ar-NO-2,6-Me$_2$, Los-Pyr-NO** and **Los-NO** exhibited a greatly reduced potency index (about two orders of magnitude). Finally, compound **LosAr-NO-α-Me** showed only partial vasorelaxing efficacy (< 50 %, at the highest concentration administered), which did not allow the calculation of the potency index.

The experimental results emerging from the time-course protocols are fully consistent with the pIC_{50} values recorded with the cumulative concentration-response curves, thus indicating that the differences in the potency are linked to different rates of NO release. As expected, the concentration of 1 μM of compounds **LosAr-NO-α-Me, Los-p-Ar-NO-α-Me, Los-Ar-NO-2,6-Me$_2$, Los-Pyr-NO, Exp-Phen-NO** and **Los-NO** evoked modest vasorelaxing responses (< 25%) in this experimental protocol, because of their low potency indexes. Compound **Los-Ar-NO** (1 μM) determined a reduction to approximately one half of the contractile tone of the vessels (Figure 23). The most potent compound **Los-p-Ar-NO** (1 μM) caused an almost complete vasorelaxing effect (Figure 23). Although compounds **Exp-Benz-NO, Exp-p-Benz-NO** showed vasorelaxing potencies lower than that of **Los-p-Ar-NO**, their time-course vasorelaxing profiles were similar to that of **Los-p-Ar-NO** (Figure 23). Moreover, a comparison of the time-course vasorelaxing effects of the synthesised compounds with those of sodium nitroprusside (SNP, a rapid NO-donor selected as a reference drug) showed that the release of NO by NO-sartans is a slow process (Figure 23).

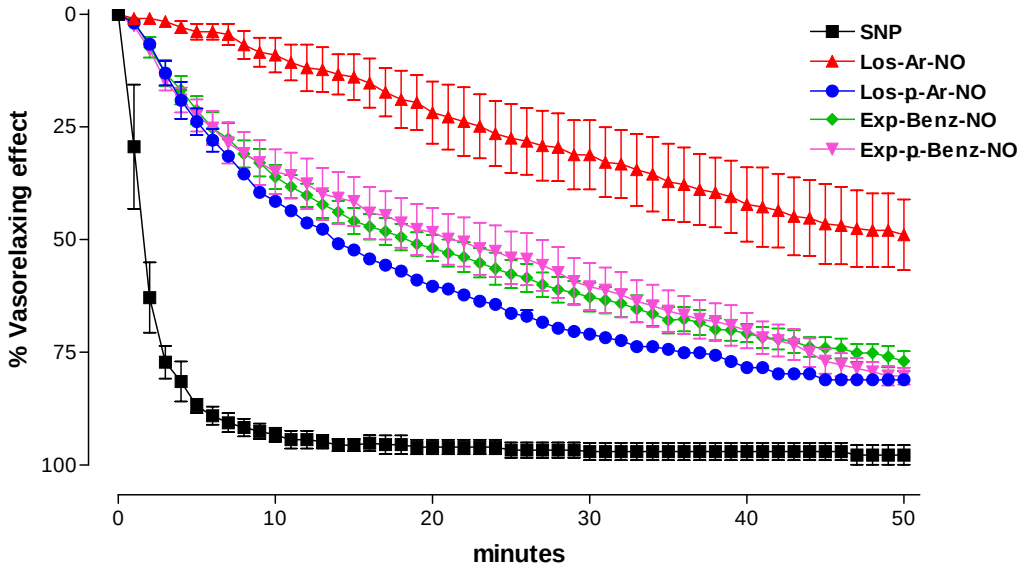


Figure 23. Representative time-course profile of the vasorelaxing effect evoked by the concentration 1μM of selected test compounds. Compounds presenting a final vasorelaxing effect lower than 25% are not shown. X Axis : time expressed in minutes. Y Axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM; Bars: standard errors.

AT1-antagonist activity

After 20 min of incubation, compounds **Los-p-Ar-NO** and **Los-Pyr-NO** exhibited AT1-antagonist properties, with potency parameters (Kb) in the nanomolar range, similar to those shown by the pioneer drugs **Los-Al-NO** and **Los-Ar-NO** and to that of the reference antagonist losartan (Table 5).

In these experimental conditions, compounds **Los-Ar-NO-α-Me**, **Los-p-Ar-NO-α-Me, Los-Ar-NO-2,6-Me$_2$** and **Los-NO** failed to exert any significant antagonism, while a prolonged incubation time (60 min) allowed compounds **Los-Ar-NO-α-Me**, **Los-p-Ar-NO-α-Me** and **Los-NO** to exhibit a significant AT1-antagonism, with potency indexes which again reached the nanomolar range (Table 5). As regards compounds **Exp-Phen-NO** and **Exp-Benz-NO, Exp-p-**

Benz-NO, hybrid derivatives of EXP3174, after a "brief" incubation time (20 min) only compound **Exp-Phen-NO** (0.1 μM) determined an almost complete abolition of the vasocontractile effects of angiotensin II, exhibiting the profile of insormountable antagonism typical of EXP3174 (Table 5). Compounds **Exp-Benz-NO, Exp-p-Benz-NO** were uneffective after a "brief" incubation time and required a prolonged one (60 min) to release the "native" drug EXP3174 and thus to exert an insormountable antagonism (Table 5).

Compounds	Kb (20min)	Kb (60min)
Losartan	4nM	9nM
EXP 3174	Insormountable*	Insormountable*
Los-Al-NO	16nM	N.T.
Los-Ar-NO	6nM	10nM
Los-p-Ar-NO	19nM	N.T.
Los-Ar-NO-α-Me	N.A.	12nM
Los-p-Ar-NO-α-Me	N.A.	8nM
Los-Ar-NO-2,6-Me_2	N.A.	N.T.
Los-Pyr-NO	8nM	N.T.
Exp-Phen-NO	Insormountable*	N.T.
Exp-Benz-NO	N.A.	Insormountable*
Exp-p-Benz-NO	N.A.	Insormountable*
Los-NO	N.A.	3nM

Table 5. Antagonist potency values of the test compounds (expressed as Kb) after a "brief" (20 min) and a "longer" (60 min) period of incubation. N.A. (no activity) means that the compound has no AT1-antagonist properties after a "brief" period of incubation. N.T. (not tested) means that the compound which showed AT1-antagonist properties after 20 min was not tested in the 60 min incubation protocol. The asterisk (*) indicates that the compound exhibited an insormountable antagonist feature, typical of non-reversible receptor antagonists, such as EXP3174.

Although the reference AT1 antagonists **Losartan** and **EXP3174**, as well as the hybrid derivative **Los-Ar-NO**, showed the antagonist properties after 20 min of incubation time ("brief" period), they were also incubated for 60 min (prolonged incubation). Their antagonist profiles recorded in these latter experimental conditions were almost equivalent to those observed after the "brief" incubation time (Table 5).

Cardiac anti-hypertrophic effects

As widely reported in international literature (Lassila et al., 2003), the spontaneously hypertensive rats used in this study previously reported (Breschi et al., 2004) showed a significant cardiac hypertrophy, almost exclusively due to an increased mass of the left ventricle (Figure 24).

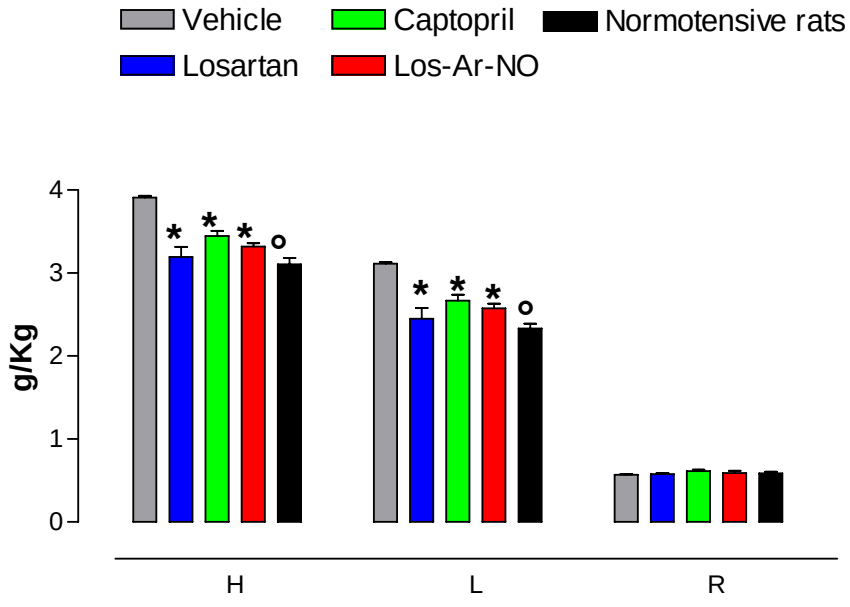


Figure 24. The histograms represent the ratios expressed in g/Kg.
[H] indicates the ratio value between the whole heart weight and the rat body weight
[L] indicates the ratio value between the left ventricle weight and the rat body weight
[R] indicates the ratio value between the right ventricle weight and the rat body weight.

Vertical bars indicate the standard error. The asterisk (*) indicates significant statistical differences between a treated group and the corresponding vehicle group. The circle (°) indicates a significant statistical difference between the normotensive group and the vehicle group.

As expected, the administration of the AT1-antagonist losartan or the ACE-inhibitor captopril led to a reduction in the left ventricle mass and, consequently, in the overall cardiac mass, which went down to the levels recorded in the normotensive animals. In the same type of experiment, compound **Los-Ar-NO** exhibited anti-hypertrophic effects, which proved to be very similar to those shown by losartan and captopril (Figure 24).

Anti-ischaemic effects

Losartan and compound **Los-Ar-NO** were tested on an experimental model of cardiac ischaemia/reperfusion.

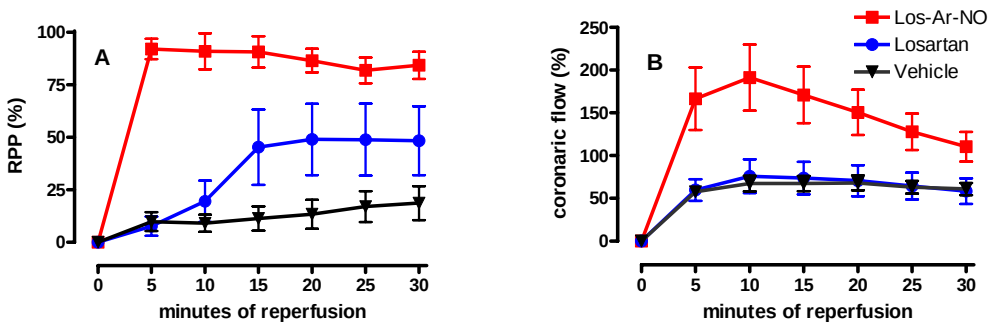


Figure 25. Parameters of RPP and CF, recorded during the 30 min of post-ischaemic period and expressed as a % of the respective values observed in the pre-ischaemic period, on Langendorff perfused hearts from rats pre-treated with vehicle, losartan or compound **Los-Ar-NO**. The vertical bars indicate the standard error.

The ischaemic injury, evaluated with the functional parameters of post-ischaemic inotropism (evaluated as Rate-Pressure Product, RPP) and coronary flow (CF), was not reduced by losartan. While compound **Los-Ar-NO** induced a marked

improvement of the two parameters: the RPP parameter reached an almost complete recovery of the levels recorded in the pre-ischaemic time and the CF recorded in the reperfusion time was almost two-fold higher than that recorded in the pre-ischaemic period (Figure 25).

Anti-platelet effects

Adenosine diphosphate (ADP) 5μM determined on rat platelet rich plasma (PRP) a significant platelet aggregation (67 ± 1 %; Fig. 26A). The effect of ADP was partially reduced by losartan (50 ± 5 %; Fig. 26A). Compound **Los-Ar-NO** caused a more pronounced anti-platelet activity, determining an about half-reduction of the aggregating effects of ADP (41 ± 4 %; Fig. 26A). This inhibition of aggregation by **Los-Ar-NO** is more evident in human PRP in which ADP 5μM determined an aggregation of 27 ± 1 % (Fig. 26B) not significantly inhibited by losartan (20 ± 4 %; Fig.26B) but significantly inhibited by **Los-Ar-NO** (12 ± 1 %; Fig. 26B).

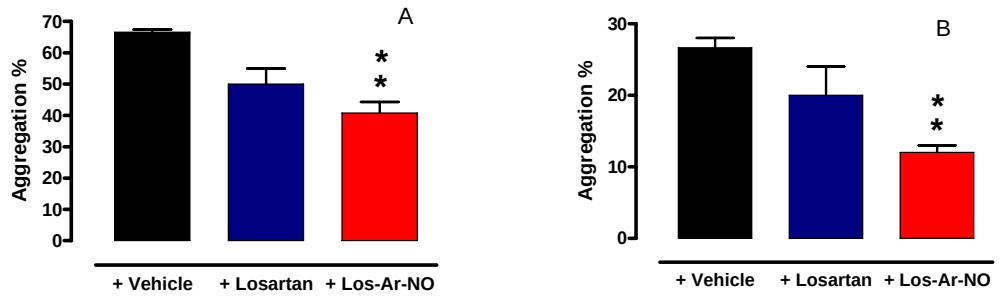


Figure 26. The histograms represent the aggregating effects induced by ADP 5μM on rat (A) and human (B) platelet rich plasma (PRP), in the presence of the vehicle, losartan or compound Los-Ar-NO. The vertical bars indicate the standard error.

Discussion

The two reported pioneer compounds (**Los-Al-NO** and **Los-Ar-NO**) showed both NO-releasing effects and AT1-antagonist properties. In particular, compounds **Los-Al-NO** and **Los-Ar-NO** produced vasorelaxing effects, showing potency indexes (pIC_{50}) in the micromolar range. These vasorelaxing effects were shown to be due to the release of NO, because they were antagonised by the guanylate cyclase inhibitor ODQ (1μM).

Both these compounds exhibited also the desired AT1-antagonist effects. As the hybrid compound **Los-Ar-NO** exerted AT1-antagonist effects in eserin-free conditions, while the presence of eserin (esterase inhibitor) dramatically reduced this antagonism, it was clear, as previously demonstrated, that the AT1-blocking activity was due to the hydrolytic cleavage of the side chain and the release of the "native" losartan. Consistently, the AT1-antagonist properties of **Los-Al-NO** and **Los-Ar-NO** (Kb = 16 nM and 6 nM, respectively) were slightly lower (**Los-Al-NO**) than, or substantially equivalent (**Los-Ar-NO**) to, those shown by losartan itself (Kb = 4 nM).

This experimental evidence demonstrated the concrete possibility of developing such a class of pharmacodynamic hybrids.

As regards hybrid "dual" molecules, the presence of both the desired mechanisms of action represents the first necessary condition. Another fundamental aspect concerns the correct balancing of the two pharmacodynamic properties, in order to obtain drugs that can profitably be applied in therapy. In particular, the NO release from an NO-sartan should be correctly modulated in order to strengthen the anti-hypertensive activity of the native drug and, in particular, to ensure

additional desired beneficial cardiovascular effects such as the antiplatelet and anti-ischaemic ones, without exasperating the negative effects, such as an excessive hypotensive response. According to this view, the NO-modulated vasorelaxing effects should be moderate, because the additional contribution of a strong vasodilator activity seems to be inessential (or even deleterious) for drugs already possessing a good anti-hypertensive efficacy, such as sartans.

Therefore, in order to develop a representative collection of prototypical NO-sartans differing from each other in their variable NO-releasing rates, compound **Los-Ar-NO** underwent various structural modifications of the NO-donor moiety. The first step consisted of the direct insertion of the nitric ester on the hydroxyl group of losartan. The target compound **Los-NO** exhibited a low vasorelaxing potency and, consistently, the administration of **Los-NO** at a concentration of 1μM did not produce any vasorelaxing effect in the time-course protocols. This can be explained by the hypothesis of a very slow release of NO and, consequently, of free losartan from the hybrid. This hypothesis seems to be confirmed by the observation of the AT1-antagonism of this compound. Indeed, **Los-NO** did not antagonise the AT1 receptor after a "brief" incubation (20 min), indicating that such a short period does not allow the hybrid to release losartan. On the contrary, a longer incubation time (60 min) allowed the compound to reach a losartan-like AT1-antagonism. Moreover, the unsatisfactory NO-releasing properties of **Los-NO** suggested the need for the presence of a "linker" moiety between the native sartan and the nitrate ester, ensuring a significant, albeit modulated, release of NO. Therefore, a series of NO-releasing side chains, analogues of **Ar-NO,** were synthesised. Preliminary experiments demonstrated

that all these side chains showed ODQ-sensitive vasorelaxing effects due to the release of NO, with potency indexes (IC_{50}) ranging around the micromolar order of magnitude.

The pyridine compound **Los-Pyr-NO**, with the heterocycle ring replacing the benzene one of **Los-Ar-NO**, showed a significant decrease in the vasorelaxing potency, and a consistent lack of vasoactive effects in the time-course protocol. Compound **Los-Pyr-NO** exhibited a losartan-like AT1-antagonist potency after the "brief" incubation time (20 min), indicating a rapid hydrolytic cleavage of the side chain and a rapidly exhaustive release of free losartan.

The introduction of (a) methyl group(s) on the nitrooxymethyl chain carbon or on the benzene ring of **Los-Ar-NO**, giving compounds **Los-Ar-NO-α-Me** and **Los-Ar-NO-2,6-Me_2** respectively, determined a negative impact on the NO-releasing properties. Indeed, the vasorelaxing potencies fell dramatically both in **Los-Ar-NO-α-Me** (pIC_{50} not calculable because of the vasodilator efficacy < 50%) and in **Los-Ar-NO-2,6-Me_2**. Of course, these two compounds were devoid of any vasorelaxing effects in the time-course protocols. Although the lack of a significant NO-releasing property makes these two compounds uninteresting for further development, their AT1-antagonist profile was also evaluated. Neither of the compounds exhibited any significant AT1-antagonism after the "brief" incubation time (20 min). Compound **Los-Ar-NO-α-Me** was arbitrarily selected (as representative of both of them) for the evaluation of the AT1-antagonism after a longer incubation period (60 min), and in these experimental conditions it showed an AT1-blocking potency similar to that shown by losartan. This evidence seems to indicate that the hydrolytic cleavage of the side chain, allowing the

release of free losartan, could be slowed down by the presence of (a) methyl substituent(s).

The shift of the nitrooxymethyl chain from the *meta* position of **Los-Ar-NO** to the *para* position of **Los-p-Ar-NO,** led to a substantial increase of vasorelaxing potency. This vasorelaxing activity was ODQ-sensitive, indicating the involvement of the release of NO. Consistently, **Los-p-Ar-NO** also exhibited the highest vasorelaxing effect in the time-course protocol, leading to an almost complete recovery of the basal tone of the vascular smooth muscle pre-contracted by KCl. However, it is noteworthy that a comparison of the time-course profiles of the vasorelaxing effects induced by **Los-p-Ar-NO** and by sodium nitroprusside, demonstrated that **Los-p-Ar-NO** can be considered as a slow NO-donor. After the "brief" incubation time (20 min), **Los-p-Ar-NO** demonstrated a clear AT1-antagonism, although it showed a potency index slightly lower than that of losartan, probably because of a significant, but not yet complete, release of the free "native" drug.

As clearly emerged from the comparison between the two analogues **Los-Ar-NO** and **Los-Ar-NO-α-Me**, also the introduction of a methyl substituent on the nitrooxymethyl chain of **Los-p-Ar-NO**, giving the analogue **Los-p-Ar-NO-α-Me**, caused a slowing down of the NO-releasing rate, represented by the lowering of the potency index. The time-course protocol revealed a weak vasorelaxing effect induced by **Los-p-Ar-NO-α-Me** 1μM. As observed in the couple of analogues **Los-Ar-NO** and **Los-Ar-NO-α-Me**, also for the couple **Los-p-Ar-NO** and **Los-p-Ar-NO-α-Me** the presence of the methyl group seems to cause a delayed hydrolytic cleavage of the side chain and a delayed release of free losartan,

because the AT1-antagonism was not recorded after the "brief" incubation (20 min), but it appeared after 60 min of incubation, with losartan-like levels of potency.

Finally, the investigation of the impact due to the "inversion" of the ester bond linking the side chain to the sartan, was performed by studying **Exp-Phen-NO** and **Exp-Benz-NO** and **Exp-p-Benz-NO**. These compounds exhibited a significant vasorelaxing potency. In particular, the vasorelaxing potency of compound **Exp-Phen-NO**, a phenolic ester, was lower than that of **Los-Ar-NO**. The insertion of a benzylic linker (**Exp-Benz-NO** and **Exp-p-Benz-NO**) led to a significant increase of the potency parameters, which resulted almost similar to that exhibited by **Los-Ar-NO**. Consistently with what observed with the couples of analogues discussed above (**Los-Ar-NO** *vs* **Los-p-Ar-NO** and **Los-Ar-NO-α-Me** *vs* **Los-p-Ar-NO-α-Me**), the shift of the nitrooxymethyl chain from the *meta* position to the *para* position (**Exp-Benz-NO** *vs* **Exp-p-Benz-NO**) caused, again, an increase of the vasorelaxing potency. As expected, the vasorelaxing effect of **Exp-Phen-NO**, at a concentration of 1μM, was negligible in the time-course protocol. While compounds **Exp-Benz-NO** and **Exp-p-Benz-NO** showed time-course vasorelaxing profiles similar to that of **Los-p-Ar-NO**.

As regards the AT1-antagonism, **Exp-Phen-NO** showed a high antagonist effectiveness after the "brief" incubation time (20 min), indicating a relatively rapid cleavage of the "inverse" phenolic ester and a complete release of active **Exp3174**. The presence of the "inverse" benzylic ester of **Exp-Benz-NO** and **Exp-p-Benz-NO** caused a slowing down of the hydrolytic cleavage; indeed,

compounds **Exp-Benz-NO** and **Exp-p-Benz-NO** showed significant AT1-antagonism (due to the release of **Exp3174**) only after 60 min of incubation time.

As the rational strategy leading to the development of a generic pharmacodynamic hybrid aims to confer additional positive properties, without decreasing the fundamental profile of effectiveness of the native drug, it is evident that such a hybrid must fit in with the "golden paradigm": it must not show any reduction of the basic pharmacological properties of the native drug itself. As far as sartans are concerned, this pharmacological class is considered as fully satisfactory for the treatment of important cardiovascular diseases, such as hypertension and/or cardiac hypertrophy. Consequently, the first inevitable step in the development of NO-sartans is a demonstration that, in such hybrids, the presence of the NO-donor property does not compromise (by possible pharmacodynamic interactions and/or chemical influences) the effectiveness of the native drugs, as regards their main therapeutic indications. In other words, the possible addition of new properties (for example, antiplatelet and anti-ischaemic effects, expected as a result of the release of NO) is subordinate to the maintenance of intact (or improved) anti-hypertensive and anti-hypertrophic effects, since their reduction can undermine the rational basis for the development of this novel class of cardiovascular drugs.

Consequently, in the light of the impossibility of identifying the NO-sartan exhibiting the optimal balance between the two pharmacodynamic properties, compound **Los-Ar-NO** was selected as a representative NO-sartan for further preliminary investigation, since it presented intermediate NO-releasing rate levels, among the various NO-sartans synthesised in this work. As previously reported (Breschi et al., 2004), the chronic oral or subcutaneous administration of **Los-Ar-**

NO demonstrated significant anti-hypertensive effects on spontaneously hypertensive rats. These effects were almost the same as those exerted by the ACE-inhibitor captopril and equivalent to (or perhaps slightly better than) those shown by the equimolar doses of the reference AT1-antagonist losartan. Furthermore, in this work it has been demonstrated that **Los-Ar-NO** also showed significant pharmacological effects in the reversion of the left ventricle hypertrophy, typical of spontaneously hypertensive rats. Again, these anti-hypertrophic effects were very similar to those exhibited by the reference compounds captopril and losartan.

Finally, important, albeit preliminary, experimental data demonstrated that **Los-Ar-NO** seems to possess additional pharmacological features, not shown (or poorly shown) by the respective "native" drug losartan. In particular, **Los-Ar-NO** exerted a significant cardio-protective activity against the myocardial injury induced by an ischaemia-reperfusion cycle. Furthermore, compound **Los-Ar-NO** was more effective than losartan in the reduction of the aggregating effect induced by ADP on rat platelets. These additional properties will be studied more in depth in future pharmacological investigations.

Another essential future experimental approach will aim to the identification, among the various NO-sartans synthesised to date, of the one(s) exhibiting an optimal balance of the two pharmacodynamic mechanisms, i.e. the one(s) possessing the most convenient NO-releasing rate (Breschi et al., 2006) .

On the basis of our findings a chinese research group carried out the synthesis and the pharmacological characterization of a NO-releasing derivative of another AT1-antagonist such as telmisartan. The new hybrid drug, named WB1106, results composed by telmisartan as native molecule and a pyridinic linker bringing the nitrooxy group conjugated by an esteric bond between the carboxylic group of telmisartan and the linker hydroxylic group.

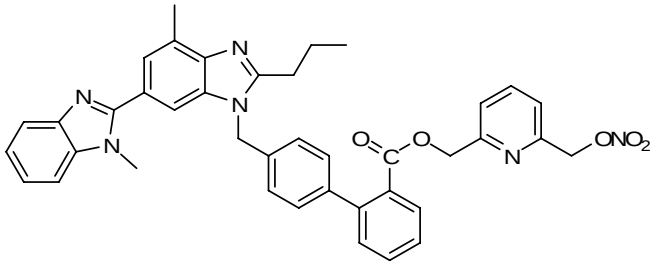


Figure 27. Molecular structure of the NO-releasing derivative of telmisartan WB1106.

The same vasorelaxing protocol carried out for the originals NO-sartans indicates the WB1106 presents an efficay value similar to its Losartan-based analogue and a potency index almost in the micromolar range. Even for this compound has been evaluated the *in vivo* anti-hypertensive activity on SHR by the tail-cuff method using the native sartan and an ACE-inhibitor as reference drug and even WB1106 like the pioneer Los-Ar-NO caused a lowering of systolic blood pressure values of SHR, leading these values near to the typical systolic blood pressure of a normotensive rat. Moreover, WB1106, in contrast to equimolar telmisartan, significantly attenuates body weight gains and improves glucose tolerance in high-fat and carbohydrate-fed rats (Li et al., 2007). These results obtained by another group confirm NO-sartans as new hybrid molecule in which the pharmacodynamic profile of the native drigs was preserved but even enriched with useful additional properties due to the NO-donor moiety.

CHAPTER 4

NO-ANTI DIABETIC DRUGS[*]

[*] The contents of chapter 4 have been already published:

PATENT PI2006A000103 "Ibridi farmacodinamici con attività ipoglicemizzante e NO-donor ottenuti dalla coniugazione di derivati idrossilati della glibenclamide con acidi carbossilici nitrossi-sostituiti" **Università di Pisa**.

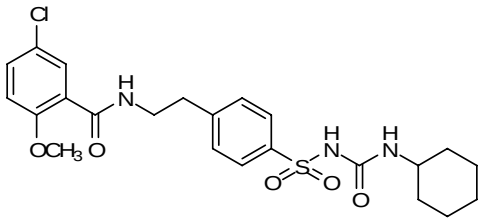
Introduction

Diabetes mellitus is a multifactorial disease associated with a number of microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (ischeamic heart disease, cerebrovascular disease and peripheral vascular diseases) complications (Virsaladze and Kipiani, 2001; Cooper et al., 2001; Clark and Lee, 1995). During the development of diabetes several biochemical (oxidative stress, modified low-density lipoprotein cholesterol, impaired NO production and chronic inflammation) and mechanical (low shear stress and hypertension) factors converge against the endothelium, resulting in endothelial dysfunction and vascular inflammation. So all these harmful stimuli associated with, and derived from, the state of insulin resistence and type 2 diabetes markedly enhance endothelial dysfunction, which determines a NO reduced biosynthesis by endothelium providing the pathophysiological basis for a great cardiovascular risk (Hartge et al., 2007).

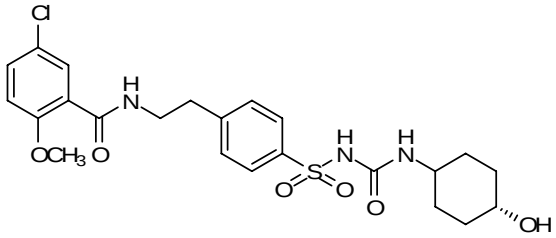
On the basis of these remaks the exigency emerged to couple the hypoglicemic activity of an insulin secretagogue such as **Glibenclamide,** commonly employed in the type 2 diabetes therapy, with the several benefical properties of NO such us the vasorelaxing, anti-platelet/anti-thrombotic and cardioprotective activities.

In order to obtain an hybrid molecule in which the NO-donor moiety is conjugated to the native drug by an *in vivo* easily hydrolizable bond such as the esteric one, **Glibenclamide,** as native drug**,** is replaced by one of the two its active metabolites, the 4-*trans*-hydroxyglibenclamide (**Gli-OH**) possesing a hydroxy group useful for the esteric bond.

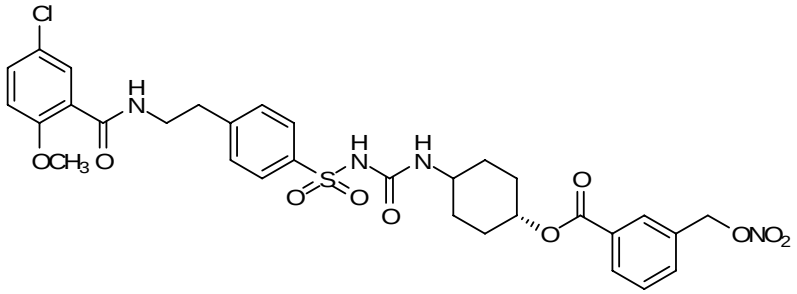
In order to modulate the release of nitric oxide, two different NO-donor linkers bringing the nitrooxy group, respectively, in *meta* or in *para* position were synthesised and pharmacologically evaluated.



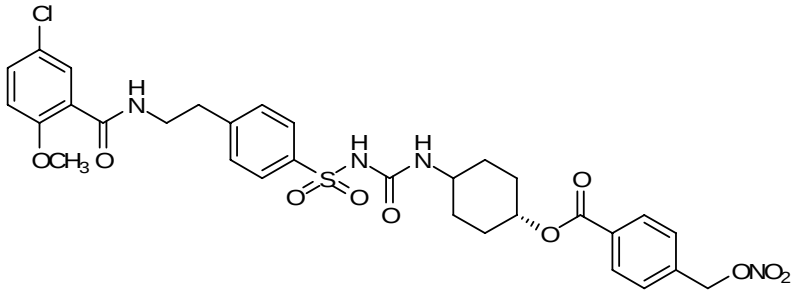
Glibenclamide



4-trans-Hydroxy-Glibenclamide



***meta*-NO-Glibenclamide**



***para*-NO-Glibenclamide**

Figure 27. Molecular structures of the reference drugs such as Glibenclamide and 4-*trans*-hydroxy-Glibenclamide and of the new hybrid compounds *meta*-NO-Glibenclamide and *para*-NO-Glibenclamide.

Chemistry

From a chemical point of view, it is possible to summarise the synthesis of the two hybrid derivatives of hydroxy-Glibenclamide as a condensation reaction between a hydroxylated derivative of the suphonylurea (such as the active glibenclamide metabolite, hydroxylated on the cycloexylic ring) and a carboxylic acid.; this synthetic route could be carried on according usual procedures, in the presence of dicyclohexylcarbodiimide (DCC) and catalytic amount of dimethylaminopyridine (DMAP), as reported in literature in R.A. Hill et al Bioorg Med Chem, 11, 2003, 2099-2113.

The two nitrooxy-derivatives of the active metabolite of **Glibenclamide** (**4-*trans*-hydroxy-Glibenclamide = Gli-OH**), underwent an in vitro pharmacological study on animal models focused to demonstrate an additional vasorelaxing NO-mediated effect conferred through the conjunction of NO-donor moiety. At the same time, these new hybrid molecules were evaluated by another *in vitro* experimental protocol on human pancreatic islets aimed to verify the preservation of the insulinotropic response.

Pharmacological procedures

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

In vitro protocols.

The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250-350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding.

The aortae were immediately excised, freed of extraneous tissues and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with an unirecord microdynamometer (Buxco Electronics).

Evaluation of the NO-releasing properties

After an equilibration period of 60 minutes, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted vascular rings. A relaxation < 10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation ≥ 10% (i.e. significant presence of

the endothelium), were discarded. In the first series of experiments, we investigated the possible NO-releasing effect of the tested compounds.

From 30 to 40 minutes after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM) and when the contraction reached a stable *plateau*, 3-fold increasing concentrations of ***meta*-NO-Glibenclamide** and ***para*-NO-Glibenclamide** (1nM-10μM) were added.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 minutes.

The same experiments were carried out in the presence of a well-known guanylate-cyclase inhibitor: ODQ 1μM which was incubated in aortic preparations after the endothelium removal confirmation.

Time-course of NO-mediated vasorelaxing effect

From 30 to 40 min after confirmation of endothelium removal, aortic preparations were contracted with a single concentration of KCl 30mM, and after a stable plateau had been reached, a single concentration (1μM) of the compounds or of the reference drug sodium nitroprusside (SNP)was added. The vasorelaxing effect of the added compounds was monitored for 50 min.

Materials.

Substances used in the experimental protocols were KCl (Carlo Erba) dissolved (3M) in Tyrode solution, acetylcholine chloride (Sigma) dissolved (0.1 M) in EtOH 95% and further diluted in bidistillated water. ODQ (Sigma) was dissolved

(1 mM) in EtOH 95% and further diluted in Tyrode solution. Compounds ***meta*-NO-Glibenclamide and *para*-NO-Glibenclamide** were dissolved (10 mM) in DMSO and further diluted in Tyrode solution.

All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicles.

Data analysis.

The vasorelaxing efficacy was evaluated as maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. When the limit concentration 10 μM (the highest concentration, which could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean ± standard error, for 5-10 experiments. Student *t* test was selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

Functional evaluation of Gli-OH and *para*-NO-Glibenclamide on human pacreatic islets.

Pancreatic islets were isolated by collagenasic digestion of the gland and following purification on density gradient.

Pancreas was freed of extraneous tissues and some portions were selected (generally the body and the tail of the gland). For the digestion the collagenase enzyme (collagenase P, Roche) was employed. Pancreatic duct was incannulated and the digestion solution (collagenase 1,5 mg/ml, dissolved in 200ml of Hanks' Balanced Salts=HBSS) additionated with 2% of human albumin, (Human-Albumin® Biagini 20%), was slowly injected to distend the tissue. After the distension, pancreatic gland was introducted in a 800ml glass becker and placed in a thermostated bath at 36,5 °C. The action of collagenase was checked after 8 minutes for the first time and then every 3 minutes observing a sample of the gland on a inverted light microscope. After about 15 minutes, the becker was removed from the thermostated bath and pancreas was continuosly stirring by sterile surgical pliers as long as the digestion was complete. Then the tissue was filtered through steel sieves ordinated according the pore width, first 400 and then 90 μm. The tissue keeped on the 400 μm filter was recuperated to continue the digestion. The tissue keeped on the 90 μm filter was recuperated with HBSS additionated with 2% of human albumine in a 2000 ml becker. The same filtration, washing and storing in HBSS solution was repeated every 5 minutes for 45 minutes or to complete organ digestion.

The purification procedure targeted the separation of the exocrine tissue from the endocrine one. The HBSS volume collected was divided in conic polypropilene

50 ml tubes and centrifuged at 250g for 2minues at 4 °C. The top layer was discared and the pellet (about 1-2 ml) was resuspended with 15 ml of a solution composed by 80% Lymphoprep (Nycomed) and 20% HBSS. On the surface of the solution 10 ml of Hanks's were placed in order to obtain the convenient garadient of density. After centrifugation at 909g for 5 minutes at 4 °C, the islets were concentrated on the interface between the Lymphoprep layer and the HBSS one where they were recuperated and again centrifuged at 909g for 2 minutes at 4°C. At the end of the procedure, shares containing about 2500 islets were suspended in 40ml of colture medium (M199 additionated with 10% of bovine serum and antibiotics), placed in plastic flasks for suspeded cell coltures (75 cm^2, Iwaki) and placed in an incubator at 27°C and 5% CO_2. The medium was replaced for the first time the morning following the isolation in order to slow down, by dilution, the collagenase action, then was replaced weekly to renew the essential colture components (Bugliani et al., 2004).

To determine *in vitro* the functionality of β-cells, the isolated islets undewent a static incubation in Krebs-Ringer-bicarbonate-Hepes solutions (KRBH), additionated with 0,5% of albumin and Glucos at the concentration of 3,3 mM and 16,7 mM a pH 7,4. Groups of islets having similar size were placed into 5 ml polypropilene tubes and pre incubated for 45 min with KRBH solution containing glucose 3,3, mM. From every tubes a share of top layer was collected in order to determine the amount of released insulin in basal conditions. Subsequently the same islets were incubated for 45 minutes with glucose 16.7 mM or with **Glibenclamide** 10 μM and 100 μM, or with the its metabolite **4-*trans*–hydroxy-Glibenclamide** 10 μM and 100 μM, or with the new hybrid drug ***para*-NO-**

Glibenclamide 10 μM and 100 μM**.** Then from every tube a top layer share was collected in oder to determine the amount of released insulin following the stimulus. The isulinaemia determination was performed with IRMA method (Lupi et al., 2002; Marchetti et al., 2002).

IRMA method is an immunoradiometric assay based on the employment of antibodies, radioactively labelled, directed versus the antigene which must be determined. For this assay polypropilene tubes with a special bottom covered with monoclonal antibodies anti-insulin were employed. After the addition of the sample, a monoclonal antibodies, labelled with with I^{125} was added. After washing, the remaining radioactivity linked to the tube represented the antigene concentration. The dosage kit used for this assay was the Insulin-IRMA (PANTEC srl) (Lupi et al., 2002; Wilson and Walker, 1995).

The described procedures were carried out in two days:

1st Day: The day before the experiment, the samples were placed at 4 °C.

2nd Day: The kit standards was prepared through the addition of fixed volumes of milliQ water and mixed with the vortex. In numbered tubes was introducted 50μl of standards or of the samples. Subsequently in every tube were added 50μl of radioactive antibody and after mixing the sample were incubated for two hours at room temperature. At the end of the incubation 1 ml of washing buffer was added. Then the buffer was sucked up through a vacuum pomp and the washing procedures was twice repeated. Every sample of the numerically raising sequence was analysed by a γ-meter for 60 seconds. The standard curve graph was analysed by the software: this graph reported on Y axis the number of shot for minute and on the X axis the concentration of the related standard sample.

Results

The reference drugs **Glibenclamide** and **4-*trans*-Hydroxy-Glibenclamide (Gli-OH),** were devoid of any significant vasorelaxing effect. On the other hand, the two hybrid compounds, ***meta*-NO-Glibenclamide** and ***para*-NO-Glibenclamide** caused concentration dependent vasorelaxing response (pIC_{50}= 5.64 ± 0.05 and pIC_{50}= 6.75 ± 0.12, respectively) which were antagonised by ODQ 1µM, indicating that the activity was really due to the release of NO (Figure 29).

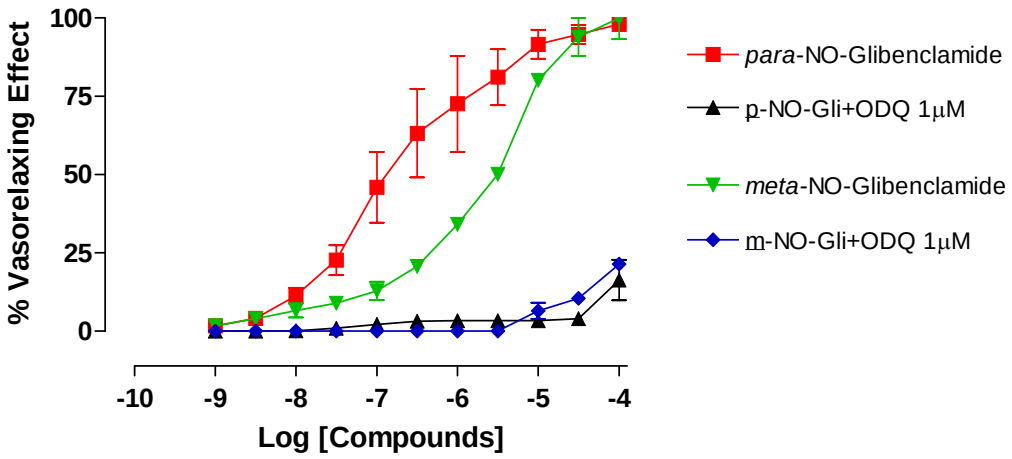


Figure 29. Concentration-vasorelaxing effect curves of selected compounds. X Axis : Logarithm of the molar concentration of the tested compound; Y Axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM; Bars: standard errors.

Consistently with the pIC_{50} values recorded with the cumulative concentration-response curves, the results emerging from the time-course protocol showed that, as already demostrated in the NO-sartans a and in the linkers time course curve, the NO-releasing rate exhibited by the hybrid that brings the NO-releasing moiety in the *para* position is higher than that exhibited by the hybrid in which the NO-donor moiety is in *meta* position.

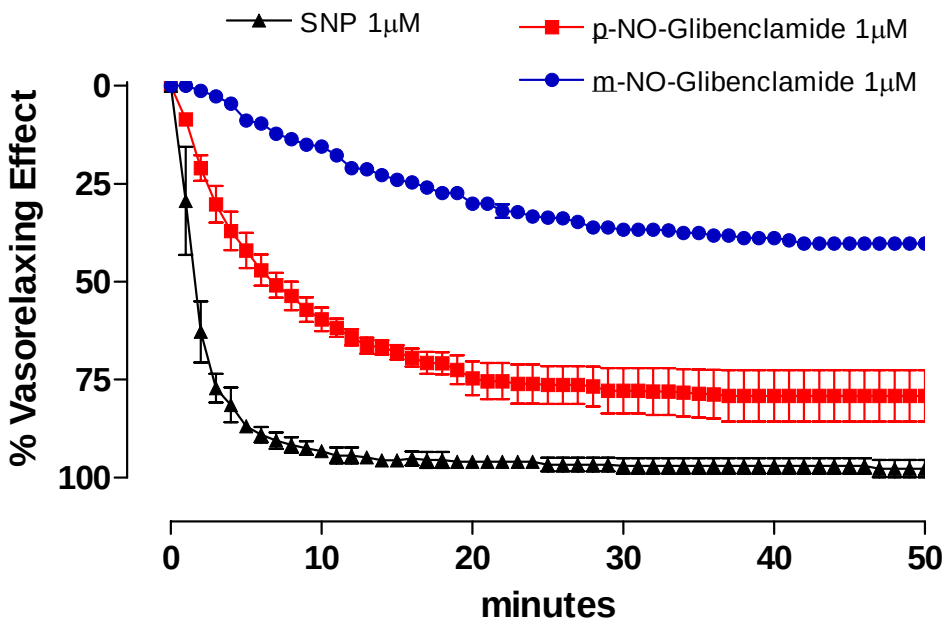


Figure 30. Time course profile of the vasorelaxing effect evoked by the concentration 1μM of the NO-anti-diabetic hybrids. X Axis: time expressed in minutes. Y Axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM; Bars: standard errors.

As concerns the evaluation of the insulinotropic effects, human islets were isolated by collagenase digestion and density gradient purification from the pancreas of 4 multiorgan donors:

Age (years): 61 ± 15;

Gender (M/F): 3/8

BMI (body mass index 25.0±4.1 Kg/m2).

Glibenclamide was choosen as reference drug.

Insulin release at basal glucose (3.3 mM) was 41.0±22.3 µU/ml.

The parameters of stimulation index are shown in Fig. 31 and Tab.6

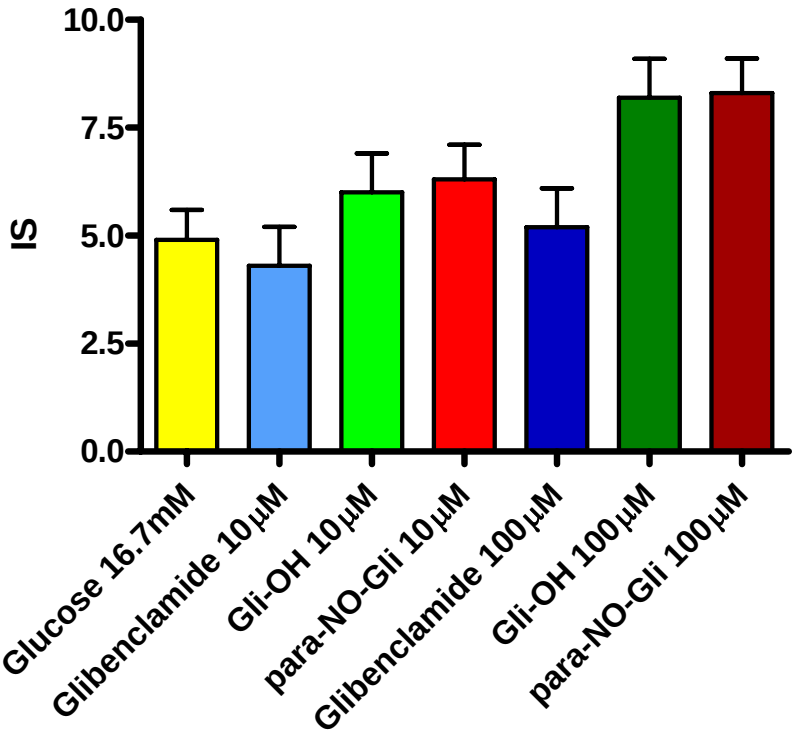


Figure 31. Insulin secretion was studied in response to high glucose (16.7 mM), **Glibenclamide** (10 and 100 µM), **4-*trans*-Hydroxy-Glibenclamide** (**Gli-OH**) (10 and 100 µM) and one of the two new hybrid compounds: ***para*-NO-Glibenclamide** (10 and 100 µM). On the Y Axis the insulinotropic activity was expressed as Stimulation Index (S.I. = ratio between the insulin release in response to a tested compound and the basal release).

Compound	Stimulation Index (SI)
Glucose 16.7 mM	4.9 ± 0.7
Glibenclamide 10 µM	4.3 ± 0.9
Gli-OH 10 µM	6.0 ± 0.9
***para*-NO-Gli 10 µM**	6.3 ± 0.8
Glibenclamide 100 µM	5.2 ± 0.9
Gli-OH 100 µM	8.2 ± 0.9
***para*-NO-Gli 100 µM**	8.3 ± 0.8

Table 6. Stimulation Indexes (S.I. = ratio between the insulin release in response to a tested compound and the basal release) of the tested compounds.

These preliminary studies showed that **Gli-OH** and ***para*-NO-Glibenclamide** possess, on human islets, insulinotropic effects comparable to that exhibited by **Glibenclamide** (See Table 6). Furthermore, ***meta*-NO-Glibenclamide** and ***para*-NO-Glibenclamide** showed NO releasing properties, which can be considered as a complementary pharmacodynamic feature, of potential usefulness for cardiovascular complications associated with the diabetic status such as endothelial dysfunction (PATENT PI2006A000103).

Discussion

As reported in literature, besides the known alteration on metabolism, the diabetic pathology leeds to several cardiovascular implications (such as micro and macrovascular complications, ischaemic heart disease) deriving from endothelial dysfuction, a pathological condition in which the disruption of vessel endothelium lees to a lack of several endothelial factors, and above all of nitric oxide with a lot of consequences on cardio-vascular system. In order to approach a multifactorial pathology such as type II diabetes, often the therapy of diabetic patients is composed by several drugs as associations of more hypoglycaemic different molecules additioned by anti-hypertensive and anti-ischaemic drugs. Moreover recent studies highlight that if the correction of glycaemia by hypoglycaemic drugs could leeds to an improvement of cardiovascular diseases associated with diabetes type II, this observation could not be applicated to some sulphonylureas, as a consequence of their mechanism of action on KATP potassium channels. In fact Glibenclamide acts as a secretagogue of insulin blocking the KATP potassium channel, so allowing the increasing of calcium concentration needed for the insulin release. But on the contrary the opening of KATP channel, at cardiac level, seems to be responsible for the pre-conditioning “phenomenon” that results in a cardioprotective mechanism.

So according with the “Hybrid philosophy”, that is : correction of adverse effects and improvement of the native pharmacodynamic profile, this new class of NO-releasing hypoglycaemic drugs seems to goal the target.

Infact the obtained results showed that the introduction of the NO-donor moiety results in a real source of exogenous NO, probably following an enzymatic

mechanism, typical of nitric esters. This assumption derives from the experimental evidence on the vascular smooth muscle where the hybrids moleculs evoked a vasorelaxing response inhibited by the block of cGMP pathway by ODQ.

As observed for NO-Sartans a tuning of the NO-donor activity seems to be important for a correct balancing between the two pharmacodynamic properties and with this target, two different hybrid with diffent NO-releasing properties have been synthesised. The ***meta*-NO-Glibenclamide** exhibited a lower releasing rate emerging from a lower potency value and a slower time-course; consistently with the NO-sartans series the ***para*-NO-Glibenclamide** showed potency indexes higher then the meta-hybrid and a time course curve that indicates a quicker NO-releasing rate. Further *in vivo* studies will identify the hybrid with the optimum balancing. Once confirmed the addition of new cardiovascular properties due to the insertion of the NO-donor moiety, the study was aimed to confirm the preservation , despite of the structural modification, of the native hypoglycaemic property. The *in vitro* study on human pancreatic islets demonstrated that, as reported in literature, the active metabolite of **Glibenclamide**, **Gli-OH** maintains the insulin secretagogue property and that this activity was confirmed even for the hybrid ***para*-NO-Glibenclamide.**

CHAPTER 5

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